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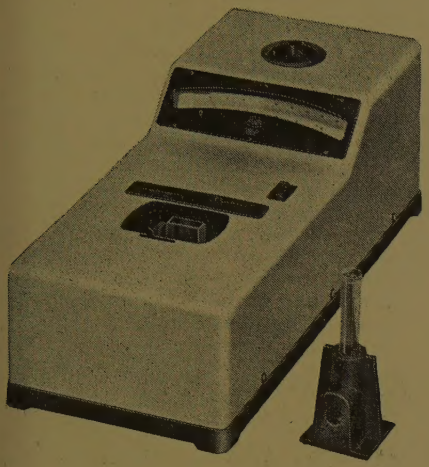

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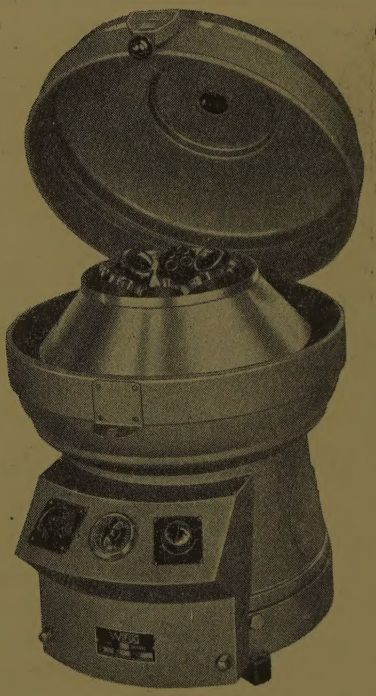

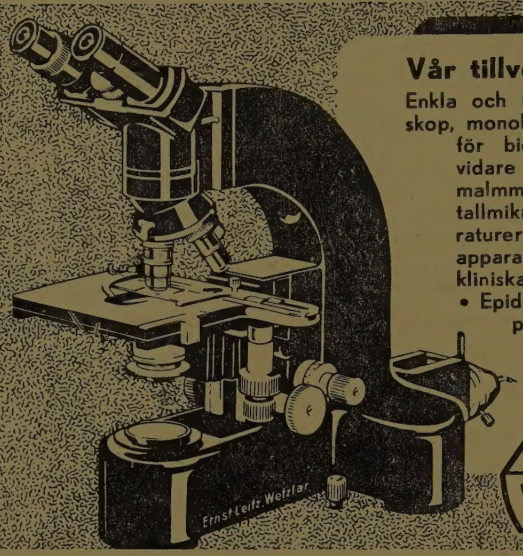

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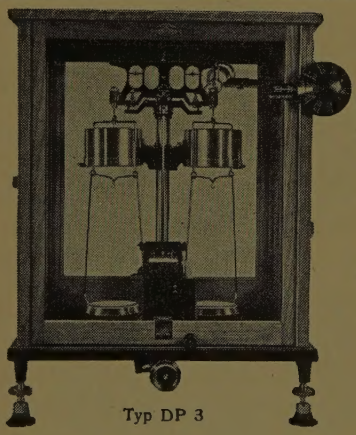

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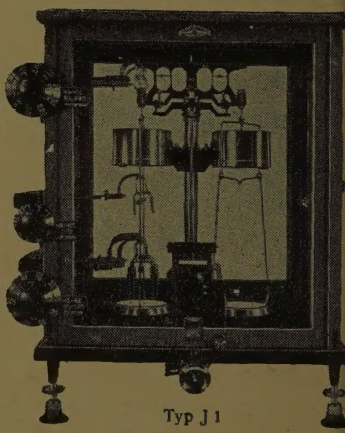

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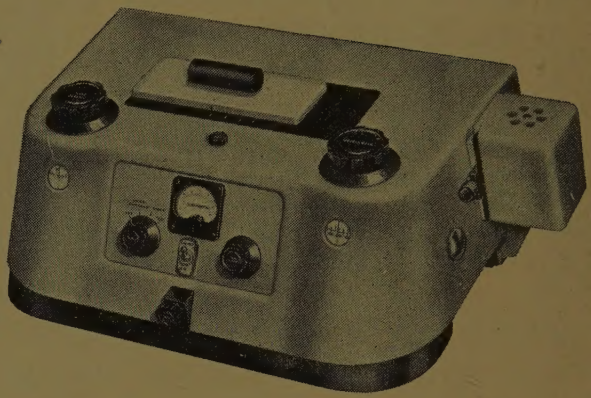

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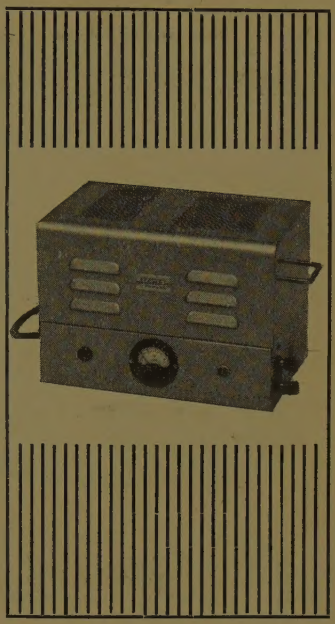

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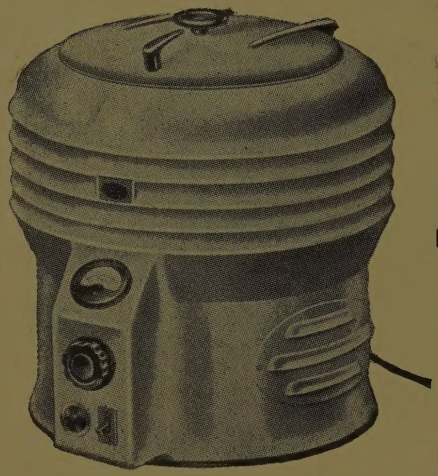

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)ETERMINATION OF THIOCYANATE IN BLOOD SERUM

By K. F. STØA

From the Biochemical Laboratory, University Clinic, Bergen

(Received for publication June 15, 1952)

For determination of thiocyanate in animal ues and fluids, colorimetric and photo- tric methods have been mostly used. The imetric or gravimetric thiocyanate deter- nations in animal material have with son, proved to be a difficult task. Rupp Schied's iodometric method (1902) also ached for by Becher and coworkers in 32, is perhaps the method which up to that e, had generally been used, at any rate urine analyses. According to Baumann, rinson & Metzer (1933, 1934) the method pears, however, to be unsuitable because, g., urine analyses may give results from to 2000 per cent too high. One of the most sitive and exact methods for colorimetric ocyanate determination is based on the ction of SCN^- with the ferric ion. Just be- se of its sensitivity, this reaction should be ecially suitable for photometric measure- nts in biological material. The ferric thio- nate reaction, however, has its definite itation, as determinations in tissue racts and biological fluids with low thio- nate concentrations are made difficult be- se of unspecific color developments (Brodie & Friedman, 1937; Lang, 1933). Lang (1933) has described the isolation of thiocyanate from biological material in the form of a complex copper-pyridine-thio- cyanate. This reaction, which was first men- tioned by Spacu (1922), is reported to be specific for thiocyanate, but hitherto appears to have been of little use, possibly because of its limited sensitivity.

Aldridge in 1944 published a method de- pending on the conversion of thiocyanate to CNBr under the influence of Br and H_2O. After elimination of excess of bromine with sodium arsenite, CNBr is allowed to react with a solution of benzidine and diluted pyri- dine. An orange to red color, usable in photometry, is developed. It is stated that HCN and HSCN could be individually de- termined by driving out HCN by bubbling air through the acid solution.

The object of the present paper is an estimation of the cyanogen bromide method, especially in comparison to the ferric thio- cyanate reaction and Lang's copper-pyridine- thiocyanate method.

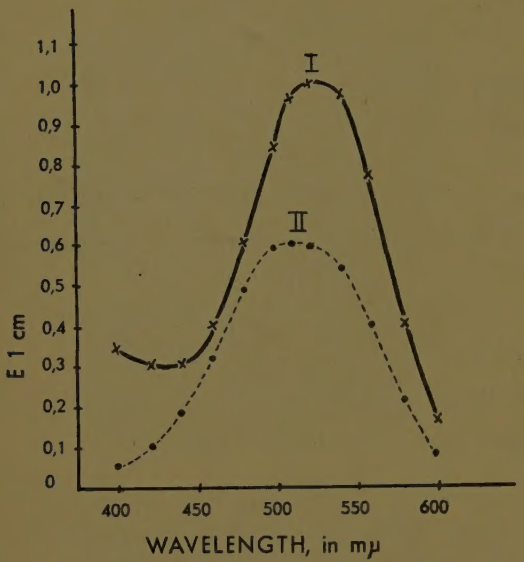

Fig. 1. Absorption spectrum of the colored solution produced by the cyanogen bromide reaction of Aldridge. Measurements in Beckman spectrophotometer.
I. The method used in analysis of serum.
II. The method used in pure aqueous solution of KSCN.

Quite apart from the study of thiocyanate concentration in serum under normal and various pathological conditions, the quantitative measurements of SCN^- in blood have importance in a number of cases. Salts of thiocyanic acid have found considerable use in the treatment of hypertension. But such a treatment presupposes that the dosage is regulated by continual serum analyses (Barker, 1936; Ginsburg & Benotti, 1939; Aas & Thingstad, 1951). Determination of extracellular fluid as described by Crandall & Anderson (1934) and later in a modified version by Lavietes, Bourdillon & Klinghofer (1936) is made by measuring thiocyanate in serum. Finally such determinations may be utilized in studying the met bolism of cyanogen compounds, as it is r cognized that HCN in the organism is tran ferred enzymatically to thiocyanate.

EXPERIMENTAL

1. *Spectral composition of the reaction color*

The spectral absorption curve of t colored solution formed by the reaction b tween cyanogen bromide and benzidin pyridine reagent is presented in Fig. 1. will be seen that the developed color has absorption maximum at about 510 mμ.

2. *Removal of excess of bromine*

The benzidine-pyridine reagent will fo a blue "quinonoid" compound with exc of bromine. Therefore the surplus brom must be removed and this is effectively do by an addition of arsenite in acid soluti The amount of bromine and arsenite m accordingly be mutually adjusted.

The arsenite used in the present expe ments is potassium arsenite, prepared in following way: 1.9 g KOH is heated w 6.2 g As_2O_3 after the addition of ab 100 ml of H_2O. When all is dissolved a the liquid cooled, the solution is diluted w H_2O to 250 ml.

The same volume of this solution as saturated bromine water must be used. Wh only half the amount of potassium arsen was used, this appeared to give great i of formation of "quinonoid" substances.

3. *Volume of bromine water*

The added volume of bromine water whit advantage be decreased to a minim i. e. 0.10 ml to 1.0 ml of deproteinized

te. This could be shown by a systematic iation of the amount of saturated bromine ter, and by investigating the influence of se variations on the color intensity of the ution formed.

n the experiments an aqueous potassium ocyanate solution containing 0.58 mg N^- per 100 ml was used. To 0.5 ml of solution an equal volume of 10 per cent eous trichloracetic acid solution was led. Bromine water and potassium arse- e in varying but mutually equal volumes re added, and finally 8.0 ml of a mixture 100 ml of pyridine reagent according to ldridge (1944) and 4.0 ml of 2 per cent eous solution of benzidine hydrochloride. ldridge's pyridine reagent consists of ml of pure pyridine, 2 ml of concentrated drochloric acid, and 73 ml of distilled ter. The solution stood for about 21 minu- before measurement in the Beckman ctrophotometer at 510 mμ.

4. *Volume of pyridine reagent*

In order to increase the sensitivity of the ction, and thereby make it more suitable analyses in blood serum, a few experi- nts were made with varying volumes of yridine reagent".

By "pyridine reagent" is meant the freshly epared acid mixture of aqueous pyridine d 2 per cent benzidine hydrochloride solu- n. The measurements were carried out an aqueous potassium thiocyanate solu- n containing 0.58 mg of SCN^- in 100 ml. ml of the thiocyanate solution, 0.5 ml of per cent trichloracetic acid solution, 0.1 ml bromine water, and 0.1 ml of potassium enite solution were used. The reaction mixtures stood 20—25 minutes for color development.

The color intensity was found to increase with decreasing volume of pyridine reagent.

5. *Composition of the pyridine reagent*

Variations in the concentration of hydrochloric acid had insignificant influence on color development within the limits 5—15 per cent. A concentration of 10 per cent HCl in the pyridine reagent ready for use was regarded as practical and used in further experiments. The concentration of benzidine hydrochloride could be varied from 0.08 g to 0.20 g per 100 ml of reagent, without clearly influencing the color reaction. A decrease of the concentration of benzidine hydrochloride to 0.05 g per 100 ml lead to less sensitivity. The pyridine concentration was also varied experimentally. Consistent with the experience of Aldridge, results proved that a pyridine content of 25 per cent in the reagent gave the highest sensitivity.

6. *Stability of the pyridine reagent*

According to Aldridge (1944) the benzidine hydrochloride must be added separately before each test as the composite reagent rapidly darkens. I have not been able to confirm this observation. The composite pyridine reagent was found to be stable for at least two weeks in my experiments.

7. *Speed of color development*

On the basis of the experimental data presented above, the following modification of Aldridge's cyanogen bromide method was employed for thiocyanate determinations.

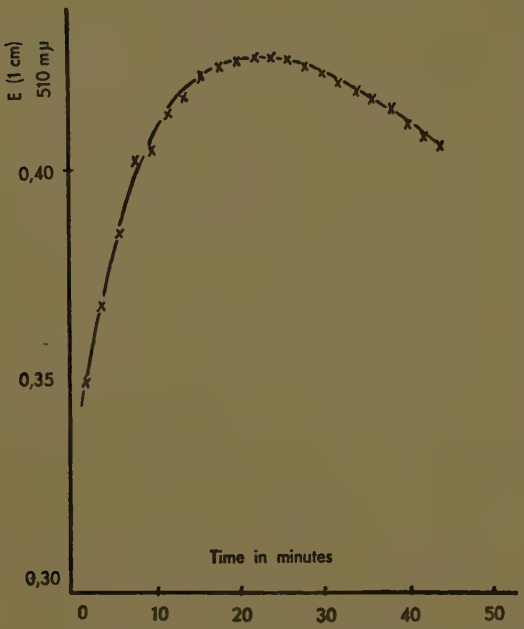

Fig. 2. The color development of the cyanogen bromide method followed by measurements in Beckman D U Quartz spectrophotometer. The reaction performed with a solution of KSCN, 0.58 mg SCN^-/100 ml.

To 1.0 ml of thiocyanate solution is added 3.0 ml trichloracetic acid 5 per cent, and the mixture filtrated after shaking. To 1.0 ml of the filtrate is added 0.1 ml bromine water, after shaking 0.1 ml potassium arsenite solution, and finally 3.0 ml pyridine reagent. The pyridine reagent used contains 0.20 g benzidine hydrocloride, 10 ml 10 per cent hydrochloric acid and 25 ml pyridine in 100 ml aqueous solution.

By this modification of the method, the progress of the color reaction was followed in the photometer for 44 minutes. The light absorption of the color solution at 510 mμ displayed maximal values about 24 minutes after addition of the pyridine reagent. The color formed is not stable (Fig. 2). It apparent from the curve in Fig. 2 that t measurements should take place 24± 3 min tes after the addition of pyridine reage The speed of the reaction depends on t temperature. Increase of temperature prov to hasten the reaction, but at the same ti the pigment appeared to be less stable. "incubation" of the reaction mixture at higher temperature does not therefore pr sent any practical advantage. Irradiati tests showed that light has no significa influence on the color development.

8. *Calibration curve*

Measurements carried out in argent metrically adjusted aqueous solutions potassium thiocyanate, proved that the re tion follows Lambert—Beer's law (Fig. The specific extinction coefficient for SCN $E\ ^{1\ mg/100\ ml}_{1\ cm}$ could in practice be set at *0.8*.

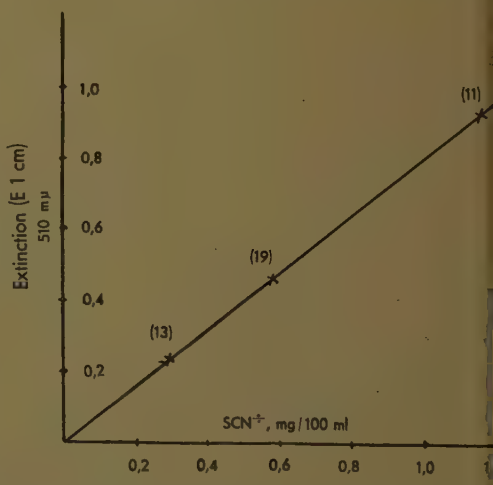

Fig. 3. Calibration curve for determinations of th cyanate by the cyanogen bromide reaction. Meast ments in Beckman D U Quartz spectrophotome Number of separate analyses in parenthesis

ble I. *Experiments with addition of µg SCN⁻ per 1 ml (as KSCN) to four ples of the same serum. SCN⁻ concen- ion before the addition: 2.3 µg per ml.*

ple	Total concentration of SCN^-, µg per ml.	Recovered µg per ml	Per cent
	6.6	4.3	97.7
	6.6	4.3	97.7
	6.5	4.2	95.4
	6.7	4.4	100.0

9. *Accuracy of the method*

he usefulness of the cyanogen bromide hod was tested by addition of known unts of KSCN to serum samples. The lts show that the method works with sfactory accuracy (Table I). The same vident from results of 10 single analyses ied out in one and the same serum ble II).

Sensitivity in comparison with the ferric thiocyanate method and the copper-pyridine method

alibration curves for the ferric thio- nate method and the copper-pyridine hod, worked out with known amounts CN, are plotted together in Fig. 4 with calibration curve for described modifica- of the cyanogen bromide reaction.

he ferric thiocyanate reaction has been ied out according to the method of Cran- & Anderson (1934) in the authors modi- tion (1952). The copper-pyridine reaction arried out as described by Lang (1933).

11. *Specificity of the method*

s stated by Aldridge, cyanide ions give nogen bromide in the same way as thio- nate. A cyanide content in the blood

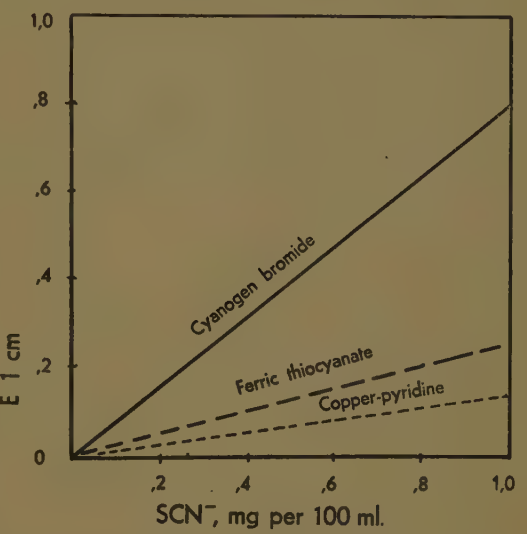

Fig. 4. The sensitivity of the cyanogen bromide method in comparison with two other methods for determination of thiocyanate. Measurements in Beckman spectrophotometer.

would therefore lead to erroneous results in serum thiocyanate analyses. However, as cyanide in acid solution may be removed

Table II. *Calculation of standard error on the basis of 10 determinations with the cyanogen bromide method in the same serum.*

Sample	Extinction (E 1 cm)	Concentration of SCN^-, mg/100 ml
1	0.190	0.24
2	0.186	0.23
3	0.183	0.23
4	0.187	0.23
5	0.187	0.23
6	0.198	0.25
7	0.180	0.23
8	0.176	0.22
9	0.178	0.22
10	0.180	0.23
		Average: 0.231

Standard error: 0.0028 (1.2 per cent of the mean value).

Table III. *Experiments with and without air bubbling through trichloracetic acid deproteinized serum filtrate.*

Serum sample	Extinction (E 1 cm)	
	No air bubbling	Air bubbling
1	0.180	0.153
2	0.124	0.119
3	0.203	0.194
4	0.162	0.163
5	0.255	0.237
6	0.426	0.447
7	0.458	0.445
8	0.436	0.456
9	0.426	0.456
10	0.146	0.150
11	0.284	0.269
12	0.230	0.238
Average:	0.278	0.277

quantitatively by bubbling air through the liquid, leaving the thiocyanate unchanged (Aldridge, 1944), it should be possible to guard against such an error by bubbling air through the filtrate deproteinized by trichloracetic acid. Such a bubbling with air was carried out in a number of serum samples. 15 minutes bubbling of air saturated with water vapor was used as a result of experiments with known pure cyanide solutions. Air bubbling does not lead to any traceable change in the results (Table III).

The absorption spectrum of the color which forms when the cyanogen bromide reaction is carried out in trichloracetic acid serum filtrate shows good specificity (Fig. 1).

CONCLUSION

On the basis of the above experimental results, the following routine method has been worked out for determinations of thiocyanate in blood serum.

To 1.0 ml serum add 3.0 ml 5 per cent trichloracetic acid solution and filter after shaking. To 1.0 ml of the filtrate add 0.1 ml bromine wa and, after shaking, 0.1 ml 3 per cent potassi arsenite solution. Add then 3.0 ml pyridine reag containing 0.20 g benzidine hydrochloride, 10 10 per cent HCl and 25 ml pyridine in 100 aqueous solution. After addition of the pyrid reagent, the reaction mixture stands at room te perature for 21—27 minutes and light absorpt is thereafter measured at 510 mμ. Distilled wa is used as a blank. A control solution in triplic is measured together with the test and also w water as blank. The control solution consists 1.0 ml H_2O treated in exactly the same way the test. The average extinction of the cont solution is subtracted from the extinction of test. The result is read off directly from the c bration curve.

This method for determination of th cyanate in blood serum has shown suffici accuracy in addition tests as well as by c culation of the standard error (Table II).

By systematically varying the differ factors which form part of the reaction has been possible to attain a maximal sens vity — a sensitivity which to a considera degree exceeds the sensitivity of the meth worked out by Aldridge. The volume bromine water and arsenite used is stron reduced. To attain greater safety agai formation of "quinonoid" compounds, concentration of arsenite solution is doubled. The amount of pyridine reag could on the other hand with advantage decreased. On several other points also, described modification of the cyanogen b mide method displays substantial deviat from the procedure of Aldridge.

In comparison with other methods ger ally used for determinations of thiocyan the cyanogen bromide method is shown to superior as regards sensitivity (Fig. 4). W the concentrations of thiocyanate norm found in human serum (Støa, 1951),

nod appears to be the only one which is itive enough to give sufficient accuracy spectrophotometry. In this case, even ic thiocyanate is not sensitive enough. measurements of higher concentrations niocyanate in serum, as with control ana- s in thiocyanate medication, the last men- ed method is indeed preferable on account ts greater simplicity.

he cyanide and thiocyanate ions give, as ed, quite identical color reactions. Experi- ts have, however, shown, that in practice can, as a rule, ignore analytical errors sed by the presence of free cyanide ions erum (Table III).

he absorption spectrum of the color de- ped in deproteinized serum shows good ificity. Sera show, however, a tendency ive a color tone with increased absorption ne blue field (about 400 mμ) (Fig. 1). It t also be mentioned, that in a very few s sera from patients develop a rather pecific color, making a sufficiently exact surement impossible. The cause of this pecific coloring is not yet clear. A similar pecific color reaction takes place when method is applied to urine. The cyanogen nide reaction, therefore, cannot without her adjustment be used in urine analyses.

SUMMARY

he cyanogen bromide reaction, described Aldridge in 1944, has been made the ct of experimental investigation in order st its applicability for thiocyanate deter- ations in blood serum. The purpose of study has further been an evaluation of reaction as compared with other methods for determination of thiocyanate in blood. By systematically varying the different factors of the reaction, it has been possible to attain a sensitivity which makes the method sufficiently exact for spectrophotometric determinations of thiocyanate in normal sera. The methods hitherto used in analyses of normal blood, appear to lack accuracy and sensitivity. Thiocyanate and cyanide both react with bromine giving cyanogen bromide. Experiments have shown, however, that cyanide ions, as a rule, may be ignored as a source of error in serum analyses.

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THE BASE-BINDING POWER OF SERUM PROTEINS AND THEIR FUNCTION AS OSMOREGULATORS IN BODY FLUIDS

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yponatremia, with corresponding reduc- of the total bases, is a frequent cal finding, which may occur without omitant dehydration. Within the limits hysiological pH a corresponding reduc- of anions must take place. The total s may be determined with great accuracy, ıot the total acids. According to Gamble 7) the average normal values are as fol-: Cl: 103 mEq., HCO_3: 27 mEq., O_4: 2 mEq., SO_4: 1 mEq., organic acids: Eq., protein: 16 mEq. The base equi-ıt of protein is usually computed by ıs of some formula. The value of organic is generally obtained by subtracting the determined + the protein from the total s.

ıe following Table shows the results of stigation of the acid-base equilibrium in al patients with different disorders h frequently lead to reduction of total . Most of them were suffering from osis of the liver, with or without ascites, heart diseases or protracted febrile con-ıs that ended in cachexia. The total have been determined electrolytically [1] by means of Hermann Nielsen's apparatus (1940), the serum proteins after Howe, chloride and bicarbonate after Van Slyke and phosphate after principle of Fiske & Subbarow.

METHODS

The protein base has been computed from the formula adopted by Van Slyke and associates (1928). The sum of Cl, HCO_3 and the protein is subtracted from the total bases. The rest anion (the column R. A. in the Table) accounts for SO_4, PO_4 and organic acids. This rest anion usually varies comperatively little. In the great majority of the cases assembled in the Table the R. A. is seen to be remarkably low, or even negative. There is no reason to suppose that these acid anions were in reality materially reduced in the cases investigated. On the contrary, an increase has been noted of the organic acids in cases of chronic disorders, cardiac insufficiency (Jervell, 1928) and in liver diseases (Hochrein & Meier, 1928, Jervell, 1928 and Schumacher 1928). The low or negative value for R. A. given in the Table can therefore not be indicative of a real acid excess, but must be due to an error. In earlier investigations of the acid-base equilibrium a similar apparent excess of acids was occasionally noted (Denis, Peters & Man, 1934 and Sunderman, 1931). Peters

[1] Magnesium will be only partially determined is method, but this has no material impor- The average value in normal material was ± 2.3 mEq., or only very slightly below what een found by other methods.

and Man assume that this may be due to the fact that the Cl is not entirely dissociated, but is in part bound to the lipoids in complex manner. They found some degree of correlation between high serum chloride and acid excess in cases of lipemia. Some of the Cl could be extracted with ether. According to Peters, the average content of lipoid Cl is 4 mEq., but he states that it may be a good deal higher. It is, however, not probable that the "acid excess" in the cases entered in the Table can be satisfactorily explained in this manner. In most cases the content of lipoids was not increased. In cirrhosis of the liver (Adler & Lemmel, 1928), infections (Denis, 1917, and Kipp, 1920) and cachectic conditions (Bloor & Knudsen, 1917) the cholesterol content in serum is usually found to be reduced. If the total content of phosphate, sulphate and organic acids together is put at 9 mEq., an "acidexcess" of up to 21.8 mEq. is found (case 14). From the Table it also appears that the R. A. varies considerably, in cases 9 and 11 showing a variation of ca. 13 mEq. in the course of a few days. This variation may take place without demonstrable alterations in the clinical condition and without change in the content of serum cholesterol. The only satisfactory explanation is that the base equivalent of the proteins is incalculable and variable. In the last column in the Table (P. B.[1]) the base equivalent of the proteins is computed by subtracting from the total bases the acids determined, with addition of 9 mEq. for sulphate, phosphate and organic acids. It is seen that the values then vary, sometimes considerably, from those found by use of the formula. There are also great variations within a short period in the same patient, without material changes in albumin and globulin content. This wo seem to indicate that the serum proteins m have a highly varying base-binding capac quite irrespective of quantitative chan The base-binding power of the proteins be altered through change of the plasma (large deviations *in vivo* are precluded), through displacement of the isoelectric po

In a case of cirrhosis of the liver (No. the following figures for the acid-base e librium were noted: CO_2: 45.5 mm, p 7.40.

Cl	98	mEq.
HCO_3	27.5	»
PO_4	0.9	»
	126.4	mEq.
Total bases	138.1	m
Determined acids	126.4	
Undetermined acids	11.7	m

The undetermined acids are in this sulphate, organic acids and proteins. cording to the formula the base equiva of the proteins, taken alone, is 25.4 m By potentiometric titration the isoele point for the proteins was found to be 7. must be assumed that in this case the teins were not dissociated, or only to a slight degree. It may even be supposed a change of the isoelectric point of the teins occurs, causing them to act as l In four cases (Nos. 9, 10, 15, 28) of diffe diseases negative values were found fo protein base, reckoned by the subtra method. The lowest value was —4.3 m It is extremely probable that in this, and haps some other cases, the proteins are

Table I.

	Date	T. B.	Cl	HCO_3	Alb.	Glob.	N.P.N.	B. P.	R. A.	B. P.[1]	Remarks
	27/5 1944	150	98.4	25.7	3.37	1.99	24.3	14.3	11.6	16.9	Leukemia acuta. Feverish
rs	16/6	145	101.4	28.2	3.42	1.69	23	12.6	2.8	6.4	from middle of May. Died 26/6.
	10/8 1943	145	98	24	3.04	1.68	16.5	11.5	11.5	14.0	Lymphogranulomatosis
rs	27/10	126.8	92.3	22.4	1.88	1.24	47.8	7.5	4.6	3.1	malign. Febrile for several months.
	17/4 1944	144	96.9	32.4	2.80	4.00	27.8	15.2	÷0.5	5.7	Tub. pulm. Febrile for
ars	22/4	142.6	95.4	32.2	2.79	4.03	27.4	15.2	÷0.2	6.0	about 3 months.
	27/4	144.0	98.5	32.6	2.44	3.96	22.6	14.1	÷1.2	3.9	
ars	26/2 1944	137.2	90.1	30	2.15	4.67	56	14.7	2.4	8.1	Tub. pulm. Febrile for several months.
	12/4 1943	145.1	95.8	26	2.90	3.63	21.2	14.8	8.5	14.3	Spondylitis c.abscessus
rs	12/10	147.2	106.5	24	3.15	3.89	24	16.0	0.7	7.7	Anemia. Periodical high fever.
rs	20/4 1944	138	94.4	26.5	2.55	4.54	23	15.6	1.5	7.7	Abscessus pulm. Febrile 4—5 weeks.
rs	16/3 1944	147.2	101.4	32	4.03	2.64	30.4	15.9	÷2.1	4.8	Abscessus pulm. Last 2—3 months afebrile.
	24/10 1943	156.6	102.4	25.6	3.12	2.20	39	12.7	15.9	19.6	Infiltratio pulm. Febrile
rs	22/11	145.7	102	28.6	3.16	2.84	43.8	14.0	1.1	6.1	about 5 weeks Afebrile from 18/11.
	14/4 1944	136	97	28,9	2.42	2.54	35.4	11.4	÷1.3	1.1	Endocartitis lenta. Febrile
rs	4/5	129.4	102	22.7	2.66	2.51	26	12	÷7.3	÷4.3	several months.
	15/5	138.2	96.4	24.2	2.50	2.80	26.5	12	5.6	8.6	
	2/5 1944	143.4	102.6	32	3.87	2.72	24.3	15.7	÷6.9	÷0.2	Polyserositis. Periodically
rs	4/5	146.3	96.4	29	4.35	2.90	21.7	17.4	3.5	11.9	febrile for several months.
	27/6	146.8	91.6	31.1	3.51	3.69	36	16.6	7.5	15.1	
	12/2 1944	147	97.6	22.3	2.96	2.72	58.2	13.2	13.9	18.1	Diabetes. Ascessus .Fever
rs	22/2	133.3	96	25.2	2.69	2.42	23	11.9	0.2	3.1	from 18/1—22/2. Blood trans-
	13/3	136.9	97	20.5	2.43	2.76	29.9	11.9	7.5	10.4	fusion. Died 17/3.
	15/3	140.8	97	31.6	2.57	2.71	31.7	12.1	0.1	3.2	
ars	11/4 1944	148.5	104	26.9	4.09	2.31	24.7	15.5	2.1	8.6	Colitis ulcerosa. Very slightly subfebrile.
	21/5 1943	136.1	101	24	2.69	3.16	23	13.1	÷2	2.1	Cirrhosis hepatis. Last 1½
ars	25/5	149.2	101.4	25.3	3.14	3.63	20.8	14.4	8.1	13.5	years ascites. 21/5 Laparo-
	4/4 1944	144.8	106.8	26.7	2.60	3.78	25.6	14.2	÷2.9	2.3	centesis 3500. From 21/5 to
	2/5	139.5	84.4	33.4	2.22	3.68	32.5	13	8.7	12.7	25/5 supply of 5 g NACl
	11/5	133	87.8	32.3	2.10	3.31	20.8	12	0.9	3.9	daily. 2/5 Laparocentesis 5000.

cont.

Case	Date	T. B.	Cl	HCO_3	Alb.	Glob.	N.P.N.	B.P.	R. A.	B. P.[1]	Remarks
14.											
B. N.	11/10 1943	139.1	94.4	29.2	2.22	10.48	25.5	25.9	÷10.4	6.5	Cirrhosis hepatis. I
W. 18 years	28/10	138.1	98	27.5	2.58	9.66	14.8	25.4	÷12.7	3.6	tes. Icterus for s
	25/1 1944	139.2	100.8	23.3	2.44	8.16	26.5	25.9	÷10.8	6.1	months. Febrile las
	24/2	147.3	102	24.1	2.61	7.76	33.4	21.8	÷0.6	12.7	Dec. Several febrile
	2/3	149.3	106.2	22.9	2.28	7.07	21.5	19.6	0.6	11.2	Febr.—March.
	17/5	142.5	105	20.9	3.05	6.75	28.7	21.1	÷4.5	7.6	
15.											
H. S.	27/7 1944	139.5	104	27.5	2.34	4.36	16.9	14.6	÷6.6	÷1	Cirrhosis hepatis.
M. 50 years	3/8	143.9	100.8	37.4	1.89	4.26	19.1	13.2	÷7.3	÷3.3	ascites. No icterus
16.											
E. L.	28/3 1944	144.4	100	27.4	2.86	6.30	20.4	20	÷3.0	8.0	Cirrhosis hepatis.
M. 36 years	31/3	144.2	99.6	21.1	2.64	5.76	29.9	17.3	0.2	8.5	several month. No
17.											
A. A.	26/5 1944	141.8	103	28.4	2.35	5.41	33.8	16.6	÷6.2	1.4	Cirrhosis hepatis. I
W. 19 years	3/6	136.6	91.6	25.8	2.54	4.81	15.6	16.0	3.2	10.2	tes. Slight icterus
											ral months.
18.											
Å. S.	5/4 1944	144.5	105.8	26.7	4.28	2.74	12.6	16.9	÷4.9	3.0	Anemia hemolytica
W. 37 years											and spleen enlarg
											Very slight icterus
19.											
M. G.	4/5 1944	143.4	101.2	27.2	4.63	2.27	21.7	16.9	÷1.9	6.0	Mitral stenosis. C
W. 34 years											able heart insuffici
											several years.
20.											
G. S.	8/5 1944	141.5	108	17.7	3.94	2.81	47 8	16.1	÷0.3	6.8	Pulm. fibrosis. Car
M. 21 years											largement. Consid
											cyanosis. Polyglob
											Died 10/5.
21.											
A. A.	12/5 1944	148.3	102.6	30.1	3.45	3.58	24.9	16.1	÷0.5	6.6	Mitral stenosis. C
W. 62 years											able insufficiency f
											ral years.
22.											
A. S.	8/3 1944	143.7	99	30.5	4.06	3.73	29.7	18.2	÷4.0	5.2	Congenital cardiac
M. 28 years											ciency. Severe cya
											Polyglobulia.
23.											
O. A.	1/6 1944	149.7	101.6	28	4.20	4.00	28.2	19.0	1.1	11.1	Mitral stenosis. M
M. 22 years											insufficiency.
24.											
N. A.	10/5 1944	142.4	103.6	29	3.57	2.83	37.8	15.1	÷5.3	0.6	Hypertonia. No sp
M. 55 years	16/5	150.3	107.2	27.7	3.72	2.25	30	14.4	1	6.4	sufficiency. From
											NaCl supply daily.
25.											
I. J.	3/5 1944	150.3	113	26	2.75	2.85	32.6	12.9	÷1.6	2.3	Arteriosclerosis. I
W. 88 years											senilis. Slight ede
											legs.
26.											
J. P.	5/5 1944	134.5	101.4	22.9	2.74	2.47	17.4	12.1	÷1.9	1.2	Cancer oesophagi.
M. 71 years											siderable nutritiona
											les. Cachexia. Ed

	Date	T. B.	Cl	HCO_3	Alb.	Glob.	N.P.N.	B. P.	R. A.	B. P.[1]	Remarks
	$^{8}/_{6}$ 1944	150.3	97.8	21	2.41	5.79	87	17.5	14	23.3	Myelomatosis. Some
ars	$^{28}/_{6}$	142	90.2	28.2	2.18	4.94	122.3	15.3	8.3	14.6	cachexia. Renal cirrhosis?
	$^{5}/_{8}$	135	94.4	25.6	2.18	4.99	136	15.4	÷0.6	6.0	
	$^{1}/_{11}$ 1943	137.3	103.8	28.5	1.90	3.49	33.9	11.8	÷6.8	÷4	Fistula pancreatis. Lues
ars	$^{15}/_{11}$	143.8	105	28.9	1.56	2.78	36	9.5	0.4	0.9	III. From $^{10}/_{11}$ 10 g of NaCl daily.
	$^{16}/_{3}$ 1944	145.6	110	25.9	2.90	1.50	13.0	10.8	÷1.1	0.7	Morb. Addisoni. Symptoms
ars	$^{24}/_{3}$	153	106.1	23.7	3.42	2.00	18.2	13.1	10.5	14.2	for 6 months. $^{7}/_{3}$—$^{17}/_{3}$ Doca injections. 95 g NaCl per os.

planations: T. B.: Total bases. B. P.: The base equivalent of protein reckoned after formula. B. P.[1]: $Cl + HCO_3 + 9$), 9 m. eq. for the sum of SO_4, PO_4 and organic acids. R. A.: rest anion. T. B. – (Cl + . P.) N.P.N. is given in mg per 100 ml. All other figures are given in m. eq./L.

ly neutral or behave as base. Normally, base-binding capacity is to an essential ee linked to the albumin fraction. It is fore most likely that the albumin frac- under pathological conditions has the est power of changing its base equi- t. In some cases of cirrhosis of the liver e 14, e. g.), where the globulin fraction tirely predominant, great variations in ase values must also be found.

a long series of experiments it has been n that the distribution of electrolytes in ent body fluids behaves according to an's law of membrane equilibrium ene et al., 1931, Hastings et al., 1927 and et al., 1922). The difference in electro- then expresses the degree of dissociation e serum proteins. In an earlier paper ch, 1945) the distribution of the electro- between plasma and a protein-poor or in-free body fluid has been employed alculation of the base-binding capacity e serum proteins. This indirect com- ion of the base-binding power supported sumption of a varying capacity for base- ng in accordance with the views that have been advanced. Derrin and associates (1937), however, have maintained in a couple of papers that the distribution of the electrolytes between plasma and the fluid of the anterior ocular chamber does not behave entirely according to the Donnan equilibrium, but is also dependent on the permeability of the vascular wall.

The disorders in which the base equivalent of the proteins is especially reduced are chronic debilitating diseases, cardiac insufficiency and especially cirrhosis of the liver, both with and without ascites.

Changes of the base-binding power of the serum proteins take place without alteration of the osmotically active anions. This must be accompanied by a corresponding change in the cations, that is to say, in sodium. A reduction of the base equivalent of the proteins entails an equimolecular reduction of the total osmotic pressure in the whole of the extracellular fluid system. At the same time there must also occur corresponding changes in the intracellular fluid. Whether this takes place through diffusion of potassium out of the cells or through increase in the quantity

of intracellular fluid cannot be decided except by exact balance experiments.

In this manner the serum proteins may play a not insignificant role for the regulation of the total osmotic pressure and for the Na content in the extracellular fluid system. Comparatively large quantities of Na may thus be retained or be excreted from the organism without alteration of the fluid balance. In one case (No. 13) the content of total bases increased by 13 mEq. without change in the intake of salt. The quantity of extracellular fluid was estimated to be 17 litres, so that the retention of NaCl was about 13 g.

Changes in serum electrolytes, obviously caused by reduction of the base equivalent of the proteins, have been frequently reported in the literature, especially in cases of cirrhosis of the liver.

Godyer and associates (1950) found an increase of serum sodium up to 10 mEq. after infusions of salt water in patients with cirrhosis of the liver. Investigations have revealed a partial retention of Na through changes in renal reabsorption (Fahrnsworth et al. 1948 and Goodyer et al. 1950). Others have also found hyponatremia in cases of cirrhosis of the liver and occasionally considerable variations in the Na concentration (Eisenmenger et al. 1949, 1950, Gabuzda et al. 1950, Nilson et al. 1951). After salt restriction and tapping of ascites, a fall in serum Na has regularly been noted. A salt deficiency syndrome after persistent tapping of ascites has also been reported (Nilson et al. 1951). After ingestion of salt the serum Na increased and at the same time there was clinical improvement. Eisenmenger and coworkers (1950) have noted a reduction in serum Na up to 12.5 mEq. Together w clinical improvement in the patients th was an increase of the Na content in se and increased excretion of Na in the ur They could note variations in serum both on salt restriction and on more lib diet. Low Na, but normal Cl values h also been reported (Fahrnsworth, 194 These investigations referred to above c firm the findings and conclusions of the p sent author. Eisenmenger and associa (Eisenmenger et al. 1950) have ascribed low serum Na values to the strong tende to ascites. The ascitic fluid makes s heavy demands upon the supply of sod that a dilution in the extracellular body f must take place. But they also found hypo tremia in patients in whom ascites was c trolled by restriction of sodium. There a rise in serum Na together with impro ment in the clinical condition.

It can hardly be doubted that most c of hyponatremia in cirrhosis of the li and frequently also in other diseases, are to changes in the base-binding power of proteins. What are then the factors that fluence the dissociation of the serum p teins? In certain cases it seems as if supply of Na is of significance. An ex of salt leads to increase in the base-bind capacity and thereby to an increase in total bases (*i. e.*, in Na). This is especi true in cirrhosis of the liver (No. 13), bu the present material it is seen also in hy tension without edematous tendency (No. in subacute bacterial endocarditis (No. and in other cases (Nos. 28, 29). On other hand, restriction of salt supply, esp ally in combination with tapping of as fluid and use of mercurial diuretics, will

the base equivalent of the proteins, with esponding reduction of the Na concen- on. In most of the cases where ingestion lt leads to increase in serum Na a re- d excretion will also be found. It is not probable that a diminished excretion dium is a primary cause. In many cases rdiac or nephrogenic edema, an excess lt will not lead to increased Na concen- on in the extracellular fluid.

liver cirrhosis with ascites a distinct elation between content of serum Na and ency to ascites may be found in the same nt. Treatment which reduces the tran- tion of ascites, such as salt restriction, ings and mercurial diuretics, will also to hyponatremia *via* the serum proteins. stion of salt increases the transudation citic fluid and also the content of serum Hyponatremia need not necessarily pro- clinical symptoms. A salt difficiency rome is therefore not directly dependent he sodium content in the serum.

ariations in the base-binding power of roteins are not, however, dependent on salt supply alone. In many cases there also come spontaneous changes. Without onstrable alterations in the clinical pic- and without changes in the diet, the bases and consequently also the serum may vary considerably (Case No. 14). he base-binding capacity must be con- ned by the qualitative changes in the n proteins, irrespective of the findings uantitative analysis. It can hardly be ted that it is the isoelectric point that bile, but it is not known what factors into play in this respect. That these ions of the proteins are regulated by nocortical hormones alone is not prob- able. The influence of DOCA on the Na concentration is probably not produced through this mechanism.

There is reason to believe that variation in the base-binding power of the proteins is a relatively frequent phenomenon in clinical medicine. Dissociation of the serum proteins, therefore, plays a not insignificant role in the regulation of the serum electrolytes and it is necessary to take these matters into account in order to obtain a correct estimation of the changes in electrolyte metabolism which may appear in a large number of diseases.

SUMMARY

In a number of patients, mostly suffering from cirrhosis of the liver, cardiac insufficiency or protracted febrile disorders, investigations of the acid-base equilibrium were pursued. In many of these cases Cl and HCO_3 form so large a proportion of the total content of acids that the remaining amount of non-determined acids and of proteins is far smaller than usual. If the base equivalent of the proteins is computed in the usual manner, the sum of the anions, even without inclusion of phosphate, sulphate and organic acids, will in many cases actually exceed the content of total bases. The cause of the "acid surplus" is discussed and it is presumed that it must be due to the serum proteins having a smaller base-binding capacity than has been supposed. Under certain pathological conditions the serum proteins must be assumed to be slightly dissociated and to have little or no base-binding power. They may perhaps even behave as bases. The formulas usually employed for computation of the base

equivalent are not valid under pathological conditions.

In several of the cases entered in the Table, most distinctly in liver cirrhosis, considerable spontaneous variations are seen, in the total bases (*i. e.*, the serum Na), which may vary a great deal in the course of a short time. Salt restriction, especially ascites tapping, reduces the content of total bases in serum, while salt surplus produces an increase up to 13 mEq. per litre. Under conditions where the total bases vary greatly, the determined acids remain almost unchanged. The explanation hereof is that the base-binding power of the serum proteins varies and that the acid-base equilibrium is maintained by corresponding changes in the serum Na without alteration in the other electrocytes. What factors, besides the ingestion of salt, regulate the base-binding capacity is not known. T ability of the proteins to alter their ba binding power, which is independent of qu titative changes, is probably related to isoelectric point. In a case of cirrhosis the liver the pH of the isoelectric point the serum proteins was found to be eq to the pH of the serum.

This hitherto unknown function of proteins is of importance for the regulat of the Na concentration in the extracellu fluid and for osmoregulation. In this w a not inconsiderable quantity of sodium n be retained or excreted without any cha in the fluid balance.

An asymptomatic hyponatremia is of met with in clinical pratice. This may, many cases, be explained by a reduced ba binding capacity of the serum proteins.

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ON THE UNSATURATED COPPER-BINDING CAPACITY OF BLOOD SERUM[1]

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Abderhalden & Møller (1928) have shown that the copper present in serum is not dialysable, but probably bound to the serum proteins. Eisler, Rosdahl, & Theorell (1936) have found that in cataphoresis copper follows the albumins. All the copper is liberated by precipitation of the proteins with 10 per cent trichloracetic acid, whereas 70 per cent alcohol precipitates the copper together with the proteins (Boyden & Potter, 1937). Copper is thus evidently present in plasma in an organic form dissociable by acids.

Mann & Keilin (1938) have prepared a blue copper protein (Haemocuprein) from erythrocytes and serum of mammals, probably an albumin, containing 0.34 per cent copper. Holmberg & Laurell (1948) later isolated a copper-containing protein from serum. This protein was found to be pure on examination in ultracentrifuge. The molecular weight is 151 000 and the iso-electric point 4.4. The copper content is 8 atoms per molecule, i. e. the same copper content as found by Mann & Keilin. The name of coeruloplasmin has been suggested for this substance.

The question now arises whether coeruloplasmin is saturated with copper or whether under normal, or possibly under pathologi conditions an unsaturated copper-bind factor is found in plasma, analogous the iron-binding protein in plasma, wh normally by no means is saturated with i (Laurell 1947).

Klotz & Curme (1948) have studied binding of copper to pure serum albumi pH 4.83 and rising Cu^{++} concentrations, ginning with about 60 mg per cent. At lowest copper concentrations about 5 cent of the copper was bound to pro provided the protein concentration approximately the same as in serum. higher copper concentrations the percen of bound copper decreased, as was also case at lower pH values. The binding pro reversible. By dialysis against a copper- outside fluid, equilibrium occurred after s time between the copper concentration the fluid inside and outside the dialysis

Macheboeuf (1946), on the other h holds that the copper added is only to a small extent dialysable. He added la amounts of copper, however, na $Cu(OH)_2$ until the serum was saturate a concentration of about 1 per cent (dr

These experiments were made under ditions differing very considerably from physiological in that, among other things added quantities of copper exceeded t

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lly present in serum several hundred
They, therefore, elucidate nothing of
sible unsaturated copper-binding capa-
f native plasma.
ally, Holmberg & Laurell (1947) men-
hat copper salts added to serum react
itatively with sodium diethyl-dithio-
mate, whereas the copper normally
nt in serum does not react. They state
xact figures, however, nor indicate
ds of investigation.

e object of the present investigations
een to study whether copper added to
n can be bound in the same way as that
ally present, and if so, to what extent.
rious methods may be conceived to be
e for this purpose.
Coeruloplasmin has a blue color. It
d therefore be possible to ascertain
ner Cu-coeruloplasmin is produced from
urated coeruloplasmin by addition of
r to serum. According to the informa-
available on the intensity of the color,
s, however, too faint to afford a basis
colorimetric determination of small
nts of coeruloplasmin.
Ultrafiltration of serum after addition
pper gives a protein-free filtrate, in
the amount of copper not bound to
in can easily be estimated.
Micro-organisms exist which while
ing obtain a steadily intensifying color
ing copper concentrations in the culture
um. Non-proteolytic micro-organisms
probably only utilize the copper not
l to protein, and are therefore service-
for quantitative estimation of the latter.
4. Experiments with sodium diethyl-
carbamate. I did not obtain sufficiently good results by using Laurell's method for estimating the color reaction after addition of copper and of sodium diethyl-dithio-carbamate. I therefore elaborated a method where the carbamate-bound copper was removed by filtration through a charcoal column.

Ultrafiltration

Experiments of ultrafiltration according to Rehberg (1943) showed this method to be useless, because the membrane material employed contained fairly large amounts of copper. For the same reason investigations based on dialysis were also useless.

I have instead studied the copper-binding capacity of serum by ultrafiltration in Thiessen's apparatus (Thiessen 1923). All glass parts of the apparatus were cleansed by standing for 24 hours in a 1 : 1 dilution of nitric acid, followed by rinsing in hot tap water, and 3 times in redistilled water. The fluid did not come into contact with metal, but with a rubber packing cleansed by boiling in redistilled water. The filter used was ultrafeinfilter, feinst.

The serum passed very slowly through the filter. However after 4 or 5 hours sufficient filtrate was collected for duplicate estimations of the copper concentration.

Filtration of redistilled water revealed inconsiderable impurities only, the copper concentration of the water after the passage through the filter being 0 and 8 μg per cent. But it must be remarked that redistilled water passed through the filter at a faster rate than the serum.

The filter proved, however, capable of retaining copper. Filtrate from a pure cupric sulphate solution with 100 μg per cent Cu contained only 56 and 60 μg per cent Cu.

Table I. *Copper concentration in filtrate after ultrafiltration through Thiessen's apparatus.*

Serum No.	Serum copper	Added copper	Copper concentration in ultrafiltrate
	μg per cent	μg per cent	ug per cent
		0	0
1	127	100	55
		200	109
		0	0
2	112	100	55
		200	67
3	95	0	7
		100	93
		0	11
4	232	50	57
		100	50
		0	7
		0	11
5	237	16	21
		33	37
		67	66

Table I shows the results of ultrafiltration of normal and pathological sera. The ultrafiltrates of sera to which no copper had been added were either free from copper, or contained such minimal amounts in proportion to the serum concentration of copper that these must be supposed to be accidental impurities penetrated during the ultrafiltration. All ultrafiltrates of sera to which copper had been added contained a certain amount of copper, though in most of the experiments less than that added. The cause of this was either that some of the added copper had been linked to protein, or that it had been retained in the filter during the ultrafiltration.

This question was the more difficult to settle, because the filters differed in quality. The filter I used for the last experiment did not always give protein-free filtrate, and it could not withstand the same pressure as t other filters employed.

Since the ultrafiltrates of sera to wh copper had been added all contained lar or smaller quantities of this substance, must conclude that the added copper w present in a partially dissociated form, contrast to the natural serum copper.

The results of these experiments are, ho ever, not conclusive, because the added c per was not in all cases recovered quanti tively from the ultrafiltrate, and because was impossible to eliminate the copp retaining capacity of the membrane.

Copper estimation by means of micro organisms

According to Mulder (1939), copper be estimated by means of micro-organis *Aspergillus niger* particularly has been u for this purpose within the field of research.

The micro-organisms to be used for investigations as the present must be un to utilize protein-bound copper. *Aspergi niger* is highly proteolytic and must there *a priori* be regarded as unfit for the purp Mulder (1939) also mentions a genus bacteria, namely *Azotobacter chroococc* which, owing to melanin production, gr with a brown to almost black color, de dent on the copper content of the cul medium. All cultures of older bacteria (4 weeks) grow dark, however, even if medium is free from copper. As these teria are not protelytic, they ought t suitable for the present investigations.

As serum copper is bound to protein *Azotobacter* are non proteolytic, we ca expect copper staining in the tubes, w

b l e II. *Copper estimation by culture of Azotobacter on medium containing serum with and without added copper.*

rum	Serum in ml in 10 ml of culture medium	Copper added to serum in µg %	Melanin production			Copper content in medium in µg%		
						From serum	Added to serum	Total
	0.5	0	–	–	–	8.8 µg %	0	8.8 µg %
	0.5	346 µg %	+ +	+ +	+ +	8.8 »	17.3 µg %	26.1 »
	0.4	0	–	–	–	7.0 »	0	7.0 »
rum	0.4	346 »	+	+ (+)	+ (+)	7.0 »	13.9 »	20.9 »
pper	0.3	0	–	–	–	5.3 »	0	5.3 »
µg %	0.3	346 »	+ (+)	+	+	5.3 »	10.4 »	15.7 »
	0.2	0	–	–	–	3.5 »	0	3.5 »
	0.2	346 »	(+)	(+)	+	3.5 »	6.9 »	10.4 »
	0.1	0	–	–	–	1.8 »	0	1.8 »
	0.1	346 »	–	–	+	1.8 »	3.4 »	5.2 »
	0.5	0	–	–	–	11.5 »	0	11.5 »
perant	0.4	0	–	–	–	9.2 »	0	9.2 »
	0.3	0	–	–	–	6.9 »	0	6.9 »
	0.3	346 »	(+)	(+)	(+)	6.9 »	10.4 »	17.3 «
rum	0.2	0	–	–	–	4.6 »	0	4.5 »
pper	0.2	346 »	(+)	(+)	(+)	4.6 »	6.9 »	11.5 »
µg %	0.1	0	–	–	–	2.3 »	0	2.3 »
	0.1	346 «	–	–	–	2.3 »	3.5 »	5.8 »
rmal	1/2	0	–		–	7.8 »	0	7.8 »
	1/2	250 »	+ + +		+ + +	7.8 »	12.5 »	20.3 »
	1/4	0	–		–	3.9 »	0	3.9 »
	1/4	250 »	+ +		+ +	3.9 »	6.3 »	10.2 »
	1/8	0	–		–	1.9 »	0	1.9 »
rum	1/8	250 »	+		+	1.9 »	3.1 »	5.0 »
pper	1/16	0	–		–	1.0 »	0	1.0 »
µg %	1/16	250 »	+		+	1.0 »	1.6 »	2.6 »
	1/32	0	–		–	0.5 »	0	0.5 »
	1/32	250 »	–		+	0.5 »	0.8 »	1.3 »
rmal	0.5	0	–		–	5.4 »	0	5.4 »
	0.5	200 »	+ + +		+ + +	5.4 «	10.0 »	15.4 »
	0.4	0	–		–	4.3 »	0	4.3 »
	0.4	200 »	+ +		+ +	4.3 »	8.0 »	12.3 »
	0.3	0	–		–	3.2 »	0	3.2 »
rum	0.3	200 »	+ (+)		+ +	3.2 »	6.0 »	9.2 »
pper	0.2	0	–		–	2.1 »	0	2.1 »
µg %	0.2	200 »	+		+	2.1 »	4.0 »	6.1 »
	0.1	0	–		–	1.1 »	0	1.3 »
	0.1	200 »	(+)		(+)	1.1 »	2.0 »	3.1 »
	0.5	0		–		10.0 »	0	10.0 »
perant	0.5	200 »		+ + +		10.0 »	10.0 »	20.0 »
	0.3	0		–		6.0 »	0	6.0 »
	0.3	200 »		+ +		6.0 »	6.0 »	12.0 »
	0.2	0		–		4.0 »	0	4.0 »
rum	0.2	200 »		+		4.0 »	4.0 »	8.0 »
pper	0.05	0		–		1.0 »	0	1.0 »
µg %	0.05	200 »		(+)		1.0 »	1.0 »	2.0 »

– + + – + + color over the whole medium surface, + color along the border, (+) color at the top only.

pure serum has been added to the medium. If the copper added to serum is not bound to protein, this may, on the other hand, produce color in the tubes containing serum with added copper. The amount of free copper can possibly be estimated by comparison with standard tubes.

The culture medium employed was that used by H. L. Jensen (dextrin-glucose agar) slightly modified (1950): Dextrin 10 g, glucose 2 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, NaCl 0.2 g, $FeCl_3$ 0.1 g, $CaCO_3$ 2 g, $ZnSO_4$ 0.02 g, Na_2MoO_4 0.005 g, agar 20 g, distilled water 1000 g. The culture medium was adjusted to pH 7.4 with NaOH.[1]

In the beginning I took all the measures recommended by Mulder (1939) to minimize the copper content of the medium. This proved, however, to be not only unnecessary, the bacteria growing without staining on non-purified medium too, but even directly disadventageous, as the bacteria grew better on nonpurified medium.

It is, on the other hand, very important that the tubes are cleansed by standing for 24 hours in a strong nitric acid solution, followed by washing in redistilled water.

Preliminary experiments with addition of pure $CuSO_4$ solution to the culture medium showed increasing staining with rising copper concentration. No color was seen in tubes with no copper added.

The investigation was made with serum from normal individuals as well as serum with a raised serum copper concentration, the latter obtained from puerperants shortly after delivery. The blood was withdra under sterile conditions and centrifug after which the serum was pipetted After the culture medium had b cooled to about 50° C, serum, with without added copper, was transferred the medium. At a temperature of 50 denaturation of the protein can hardly t place, no more than a dissolution of a p sible linkage of the added copper. I used tubes which, after addition of serum, w shaken cautiously to avoid formation of bubbles before coagulation of the cont in an oblique position. Each tube contai 10 ml of culture medium, to which was ad ½ ml of serum. If more serum was ad no bacterial growth took place. Inocula was performed with *Azotobacter chroo cum* and the tubes were left at 25° Marked growth was seen already on third day, and on the fourth beginning m nin production in the tubes where s occurred at all. Reading was performed the tenth day, when color was maximal

The results of the culture experim appear from Table II. Each investiga comprised 2 or 3 parallel series in groups. In one group serum alone added, and in the other serum contai added copper in concentrations of 20 346 μg per cent, though constant for serum sample. In each series 0.5, 0.4, 0.2 and 0.1 ml of serum was added to 1 of culture medium.

No melanine production was seen in of the tubes to which serum without co

[1] My thanks are due to H. L. Jensen, Ph. D., for guidance regarding the arrangement of the experiments, as well as for several strains of *Azotobacter chroococcum* placed at my disposal.

[1] All inoculations and preparations of the ture media were carried out at Mco by S Van, mag. agro., whom I want to thank for great help.

added. *Azotobacter* thus seem unable to e the copper normally present in serum. ing of the bacteria was, on the other , seen in all the series in which serum used to which copper had been added. color faded steadily with falling concen- ons of copper. There was accordance een the colors in tubes with the same unts of serum in the parallel series.

he more intense staining of the bacteria he three latter experiments than in the former was due to the use of two dif- t strains.

wo facts complicated the experiments. was that the sensitivity of the bacteria pper declined when they were stored on bove culture medium, even if they were uently re-inoculated. The other was that copper gave far less color in the tubes re no serum had been added to the um. Serum must therefore be supposed tensify the sensitivity of the bacteria to er, as the copper concentration in blood n is far too low to account for the dif- ce. Hence the method does not allow quantitative estimation of the amount of ein-bound copper in serum.

ble II shows, however, that the concen- on of added copper in the last tube with ive copper reaction in no case exceeded erum copper concentration in tube No. 1 ining serum with no copper added ative color reaction). We may there- be justified in concluding that part of opper contained in the tubes with added er was present in a form more easily sible to the bacteria than normal serum er. There can be no doubt that it was of the added copper that gave the color ion, but it does not appear from the experiments how much of this was free. We can, however, say for certain that if any amount of the added copper is bound to protein, this does not exceed 200 μg per cent, but is in reality probably much less.

Experiments with Sodium Diethyl-Dithiocarbamate

Finally, I have tried to use the principles suggested by Laurell (1947) for estimation of the unsaturated capacity of serum to bind iron.

Holmberg & Laurell (1947) have studied the color reaction after addition of copper and sodium diethyl-dithiocarbamate to serum. They found that none of the added copper was bound to serum, but they stated no figures and gave no details regarding their procedure.

I have tried to reproduce these experiments, proceeding as follows: To 1 ml of serum I added 10—40 μl of a 10 mg per cent $CuSO_4$ solution. After 15 minutes 3 mg sodium diethyl-dithiocarbamate was added. This was followed by shaking and reading after some minutes.

Unquestionable color reaction was seen between the copper and the carbamate, but I nevertheless abandoned the method for the following reasons: 1) The color of the serum itself is so intense that even a 1 cm thick layer, using filter S 43, gives a very considerable light absorption. The fields therefore become almost black, and assessment of the increase of the light absorption taking place when carbamate reacts with free copper becomes very uncertain. The same applies where the question of assesing whether carbamate reacts with the serum

copper is concerned. 2) Serum is rarely perfectly clear, a condition that the procedure can be employed. 3) The colorimetric estimation, which otherwise in the case of copper takes place in iso-amyl alcohol, here occurs in aqueous solution, a fact which actually makes it a partially new method. At the same time an element of uncertainty is introduced by the reaction occurring in serum, while determination of the conversion factor must be carried out in aqueous solution. The results are thus not directly comparable.

These objections have led me to elaborate another method based on the principles indicated in Laurell's method B.

TECHNIQUE

Estimation of the copper linkage by filtration through a charcoal column

Copper is added to serum in the form of cupric sulphate in such amounts that the concentration is raised by 100 or 200 μg per cent. The serum is now left standing for so long that the copper must be supposed to have been bound to the serum protein, if such binding is possible. Sodium diethyldithiocarbamate is then added, which will combine with free cupric ions, if such are present. The carbamate-bound copper is now removed by passage through a charcoal column, while the protein-bound copper passes through and can be estimated quantitatively.

Reagents:

Arenaceous sand for analysis (Riedel).
Norite SX 35.
Sodium diethyl-dithiocarbamate for analysis.
Redistilled water.
Sulphuric acid, pure conc.
Nitric acid for analysis. Conc.
Perchloric acid, 70 per cent for analysis.
Sodium pyrophosphate } purified according to
Potassium carbonate } Levin Nielsen.

Preparation of the activated charcoal column

A glass tube is used, which is 30 cm long and has a lumen of 10 mm in diameter. A narrowing is made at the lower end, and the tube is closed here by a cotton plug. A mixture of 14 g arenaceous sand and 140 mg Norite SX 35 is poured slowly into the glass tube and continually pressed down against the cotton plug with a glass rod flattened at one end. The object of this procedure is to compress the mixture as closely as possible to prevent the charcoal from passing with the fluid when the column is used. Immediately before use the column is washed through with 5 ml redistilled water.

EXPERIMENTAL

To 1 ml of serum was added 10 or 20 μ of a 10 mg per cent $CuSO_4$ solution, and the serum was left for 24 hours in a refrigerator at — 8° C. Next 3 mg sodium diethyl-dithiocarbamate was added. After standing for 15 minutes 1 ml of the sample was poured through a charcoal column in two stages with ½ ml each time. The tube was now washed with three times 1 ml of redistilled water, likewise ½ ml at a time. A scant 10 minutes ought to elapse between each two pourings over the charcoal column. The first serum sample would then in the main have passed through the column; but to remove possible remains the column was washed with 10 ml of redistilled water.

The filtrate was centrifuged for 10 minutes to remove suspended charcoal, if present, after which the copper content was estimated by wet ashing, according to Levin Nielsen (1944).

The passing through the charcoal column must occur sufficiently slowly to ensure complete removal of the carbamate-bound copper. In the present experiments the total passing time was about 60 minutes.

Table III. *Copper concentration in serum following addition of copper, addition of sodium diethyl-dithiocarbamate and filtration through carbon column.*

A. Normal.

Number		1	2	3	4
Serum copper		129–131 µg%	130–133 µg%	115 µg%	115 µg%
Serum filtered after carbamate addition	No Cu added	130 µg%	128 µg%		111–118 µg%
	+ 100 µg% Cu	128 »	125–133–140 »	105 µg%	170–170–158 »
	+ 200 »	133 »	133–150 »	120–125 »	168–180 »
	Mean	130 µg%	136 µg%	117 µg%	169 µg%

B. Patients with raised serum copper.

Number		1	2	3	4	5
Serum copper		300–315 µg%	173–177 µg%	248 µg%	174–176 µg%	199 µg%
Serum filtered after carbamate addition	No copper added	302–305–315 µg%	171 µg%			
	+ 100 µg% Cu	335–295–310 »	169–177 »			
	+ 150 »			236–249 µg%	175–171 µg%	198–198 µg%
	+ 200 »	335–301 »	168–166 »	246–254 »	175–180 »	210 »
	+ 200 »	305–308 »		265 »		
	Mean	313 µg%	170 µg%	249 µg%	175 µg%	202 µg%

Number 1, 3 and 4: puerperants. Number 2: infection. Number 5: rheumatoid arthritis.

Table III (cont.).

C. Low serum copper (umbilical-cord blood).

Number		1	2	3	4
Serum copper		63–65 µg%	75 µg%	67 µg%	75 µg%
Serum filtered after carbamate addition	+ 100 µg% Cu	67–82 µg% } standing 15 min.	82–87 µg% } standing	66–68 µg%	90–72 µg% } standing
	+ 200 »	72 »	85–90 » } 15 min.	65–90 »	77–77 » } 15 min.
	+ 100 »	70 » } 24 hours in O_2 with	72–75 » } standing		78–85 » } standing
	+ 200 »	75–82 » } 5% CO_2 at 37°	78 » } 24 hours		85 » } 24 hours
	Mean	75 µg%	81 µg%	72 µg%	80 µg%

Number		5	6
Serum copper		95–100 µg%	63 µg%
Serum filtered after carbamate addition	+ 100 µg% Cu	95–100 µg% } standing	65 µg% } Cu+ added
	+ 200 »	97 » } 15 min.	68 »
	+ 100 »	} standing	63–66 » } Cu++ »
	+ 200 »	100 » } 24 hours	68–77 »
	Mean	98 µg%	68 µg%

	A+B	C
Mean of serum copper:	177 µg%	74 µg%
Mean of filtrate copper:	185 »	79 »

The results of the experiments appear om Table III. It is seen that when serum passed to which carbamate, but no copper, ad been added, none of the normally con- ined copper was removed, and that the opper values found after addition of copper nd passage through the column were not ssentially lower than that for serum copper. erum copper thus proved not to be liberated y passage through the charcoal column.

The investigation of group A, where all ad normal serum copper values, showed hat nearly all the added copper was removed n three. The last, a puerperant, displayed perfectly normal serum copper concentra- ion, although this was estimated only five ays after the delivery. A prompt serum opper fall must therefore be supposed to ave occurred after the delivery. Serum from his patient bound about 50 μg per cent of he added copper. This can possibly be con- ected with the serum copper fall, which is upposed to have occurred after the delivery.

The investigation of group B, comprising hree puerperants, one patient with rheuma- oid arthritis and one patient with infection, howed that nearly all the added copper was emoved. Thus no binding capacity was de- monstrated for copper exceeding the serum opper level.

Group C consisted of samples of umbilical- ord blood. The mean serum copper con- entration was found to be 74 μg per cent, nd the mean total binding capacity for cop- er 79 μg per cent. There was, in other words, certain difference, though hardly greater han could be accounted for by deficient re- noval of copper in the charcoal column. Besides, 79 μg per cent is also a patho- logically reduced serum copper value.

Thus, no unsaturated copper-binding capa- city could be demonstrated in serum, either in normal serum or in serum with patho- logical serum copper values. If such is pre- sent, it is at least by no means of the same relative magnitude as the unsaturated iron- binding capacity of serum.

After addition of copper I generally let serum stand for 1—2 hours at room tem- perature, and then 24 hours at — 8° C. I sup- posed that the copper would be bound under these circumstances, if a non-saturated cop- per-binding protein was present.

The experimental conditions were altered in different ways, without influencing the results. In some experiments the copper con- centration was estimated only 15 minutes after the addition of copper. I have also tried to let the serum stand for 24 hours at 37° after addition of copper. After 24 hours the pH of the serum had risen to about 8. In certain experiments I kept the serum pH constant by letting the serum stand under oxygen with 5 per cent CO_2.

Nothing certain is known of the valency of serum copper. It may be supposed to be present in cupric form, since the coerulo- plasmin is definitely blue. The valency of the added copper is hardly of decisive import- ance, if we analogize with the conditions of iron, because the amount added is so small that it will at any rate be oxidized, or reduzed before or perhaps simultaneously with a possible protein binding. However, to make sure, I performed some estimations with the added copper in cuprous form, but without finding protein binding in these instances either.

DISCUSSION

Surgenor (1949) believes transferrin to be of importance for the binding of copper in serum; but Holmberg & Laurell (1947) have shown that this is not so. They have prepared a special copper-containing protein from serum (1948).

It was confirmed by ultrafiltration that the serum copper is not ultrafiltrable. Experiments with addition of copper showed that at least some of it was ultrafiltrable, even if the concentration of added copper was low. Serum contains, in other words, no unsaturated serum protein component with great affinity to copper, in contrast to what has been found in the case of iron.

Culture of *Azotobacter chroococcum* unfortunately proved unsuitable as a method for quantitative estimation. The experiments showed, however, that if serum possesses an unsaturated copper-binding capacity, the amount thus linked does not exceed 200 μg per cent.

Finally, the charcoal filtration experiments showed that it is doubtful whether an unsaturated copper-binding component is found in serum. These experiments presupposed that serum, after addition of copper, was left standing under conditions favorable for copper linkage. I altered the standing time, temperature, pH, and valency of the copper, without this influencing the results.

The described investigations did not elucidate whether the copper in serum is found bound to one or more proteins. Possibly it is all found as coeruloplasmin. There are two possibilities in explanation of an absent unsaturated copper-binding of serum. Either the copper requirement of the organism is so small in proportion to the amounts stored in the reservoirs, and the copper-binding protein therefore always saturated; or the coeruloplasmin is an almost undissociable compound of no significance for the transportation of copper, but rather to be regarded as a kind of hemocyanine. Anyway the serum protein concentration seems to be of importance for the amount of serum copper, conditions with a low serum protein concentration are the only ones in which a low serum copper value is known in adults (Krebs 1928, Nilsson 1948).

I have had occasion to examine a patient with chronic nephritis, who was eight months pregnant (Med. Outpatient Department Rigshospitalet, Case No. 5559/46). At this stage of pregnancy none of the women in Levin Nielsen's comprehensive series (1944) had serum copper values under 200 μg per cent. This patient's serum copper was only 90 μg per cent, thus being subnormal, if anything. The total iron-binding capacity for iron was 153 μg per cent and serum iron was 74 μg per cent; hemoglobin 60 per cent and moreover, she had a pronounced hypoproteinaemia 3.9 per cent, albumin 2.2 per cent globulin 1.7 per cent. The absent serum copper rise in this patient is probably due to a failing power to produce coeruloplasmin. The total iron-binding capacity was likewise found greatly reduced, and the patient was anaemic in spite of the fact that serum iron concentration was not particularly low.

SUMMARY

It has been demonstrated through ultrafiltration that serum copper is never ultrafiltrable, whereas this is always the case with a certain amount of added copper. Culture experiments with *Azotobacter chroococcum*

hich stain while growing on copper-con-aining media, have shown that if serum pos-esses an unsaturated copper-binding capa-ity, the amounts bound never exceed 200 μg er cent. Finally, it has been shown by filtra-ion through a charcoal column after addition f thiocarbamate that practically all added opper is removed from serum, whereas serum opper passes through. The amount of serum opper thus seems to depend on the concen-ration of copper-binding protein in serum, nd very little unsaturated copper-binding apacity of normal serum is found.

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QUANTITATIVE DETERMINATION OF THE SATURATED AND UNSATURATED HIGHER FATTY ACIDS IN FECAL FAT

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The quantitative determination of the total quantity of fatty acids excreted in the feces, is sufficient to define the severity of a steatorrhea if the fat intake is known, but throws no light on its nature.

Attempts have been made in various ways to estimate the nature of the steatorrhea. The following methods have been used to this end.

a. *Separation of the fecal fat into triglycerides, fatty acids and soaps*

To differentiate between free fatty acids and soaps has little meaning, because the proportion free fatty acid/soap depends on the pH and on the Ca- and Mg-content of the feces, and the pH as well as the Ca-content of the feces can be completely independent of the fat digestion.

The differentiation of triglycerides and fatty acids + soaps can give an impression of the activity or of the amount of pancreatic lipase, with this understanding, that a splitting < 85 per cent in patients with steatorrhea points to a shortage of pancreatic lipase. If with a steatorrhea an 85 per cent or more degree of splitting is found, then we may not conclude from this that the pancreas functions well, because the splitting can be occasioned by intestinal or bacterial lipase.

(In *normal* persons a 70 per cent degr or more of splitting is found. This low val results from the presence of relatively mu endogenous fat.)

b. *Differentiation of saturated and unsaturated fatty acids*

By determining the melting point of t fat from the feces, a very rough attem has been made to distinguish between t excretion of saturated and unsaturated fat acids, based on the great difference in t melting point of the acids mentioned. practice this method until now has giv only insignificant results, because in mo cases the melting point of a mixture of ma different substances was determined.

A better way of obtaining an insight in the difference in excretion of saturated a unsaturated fatty acids, is to determine t iodine value of the fat in the feces, based the fact that iodine is bound by a dou bond. This value is thus a measure of t amount of unsaturated fatty acids.

This method has been applied many tim (Holt, Courtney & Fales; Parsons; Bloo Sperry and others) and it has shown that preference for absorption of unsaturat fatty acids exists. For various reasons lit clinical consideration has been bestowed this important observation. At first this co

lusion was based on the iodine values of the at in an arbitrary amount of feces. This nakes an estimation possible in a qualitative ense only. That is why the authors just nentioned thereupon carried out absorption ests with fats which were either wholly unaturated or wholly saturated. In this way hey could indeed confirm the qualitative esults already found. This, however, was ot working physiologically and, moreover, t was very difficult to eliminate other inluences, e. g. that of the melting point of lietary fat. This is plain from the fact that ormal persons, on a diet with tripalmitin, howed an abnormally low coefficient of aborption, for we know that a normal person as an average coefficient of absorption of 5 per cent or more, as well on olive oil, utter fat, beef fat, as on coconut fat — four ats which differ considerably as regards aturation.

A deeper insight into disturbed fat absorpion can be obtained by determining separately and quantitatively the excretion of aturated and unsaturated fatty acids, ogether with the excretion of total fat, of eople with steatorrhea fed on a normal diet, nd by calculating the coefficients of absorpion from these determinations (C. A. $_{s.f.a.}$ nd C. A. $_{u.f.a.}$). When it has been determined — or been calculated with the help of data n the literature on the composition of dietary at — how many grams of unsaturated and aturated fatty acids are contained in the iet, and it has been determined at the same ime how many grams of unsaturated and aturated fatty acids have been excreted with ne feces, then from these data the coefficient f absorption of unsaturated and saturated atty acids can be calculated in the usual nanner.

QUANTITATIVE DETERMINATION OF SATURATED AND UNSATURATED FATTY ACIDS IN FAT FROM THE FECES

When the total number of milliequivalents of fatty acids in feces is known, it is possible, after determination of the iodine value, to calculate what portion of the fatty acids is unsaturated. Then with the help of the average molecular weight of the saturated and unsaturated fatty acids, it can be calculated how many mg of each are present in the feces.

The separation and purification of the fatty acids, followed by the determination of the iodine value, takes place as follows.

An arbitrary quantity of feces is boiled with ethanolic KOH; next, this alkaline liquid which contains 75 per cent ethanol is extracted twice with petroleum ether, thereby removing sterols and other substances possibly present, which contain double bonds and which are not of an acid nature. After acidification it is again extracted twice with petroleum ether, in this case from a medium which contains 60 per cent ethanol. This extract, containing all higher fatty acids, is purified by shaking with acidified ethanol of 25 per cent, whereby the lower fatty acids, which in the previous extraction from ethanol 60 per cent had not yet remained in the lower layer, are practically entirely removed. Now a suitable portion of the petroleum ether extract is titrated with ethanolic alkali 0.1 N with thymol blue as indicator to the first change from yellow to green. After evaporation of the petroleum ether, the soap is taken up in a solution of $KBrO_3$—KBr in water and after addition of carbon tetrachloride and HCl, the amount of bromine bound is determined by back titration (modification of the method of Winkler).

Reagents

KOH solution 33 per cent by weight

ethanol 96 per cent to which 4 ‰ amyl alcohol has been added

petroleum ether (b.p. 60—80° C), purified by distillation over pure coconut fat (5 g/l)

ethanol 75 per cent
HCl 25 per cent (= 7.7 N)
HCl 1 N in ethanol 25 per cent
0.1000 N NaOH in ethanol 96 per cent
thymol blue solution of 0.2 per cent in ethanol 50 per cent
0.2000 N $KBrO_3$—KBr solution (27.835 g $KBrO_3$ + 300 g KBr dissolved in H_2O and made up to 5 l; preserved with ± 75 mg HgI_2 per 5 l)
carbon tetrachloride
hydrochloric acid 4 N
1 N KI solution + one drop Hg/l
0.100 N thiosulphate solution
1 per cent solution of soluble starch.

Apparatus

Erlenmeyer flasks of 300 ml capacity with reflux condenser
Separating funnels of 500 ml capacity
Micro-burette of 5 ml capacity (0.1 N ethanolic NaOH)
Macro-burette (0.1 N thiosulphate)
Erlenmeyer flasks of 200 ml capacity with ground glass stopper.

Procedure

Weigh out roughly a quantity of wet feces, containing about 150 mg fatty acids (5—20 g feces). Place it with weighing paper in an Erlenmeyer flask of 200 ml capacity and add 10 ml KOH 33 per cent and 40 ml ethanol 96 per cent, which contains 4 ‰ amyl alcohol. After the addition of a few chips of pumice, reflux for 20 minutes and cool. Pour the contents quantitatively into a separating funnel of 500 ml capacity and then shake twice, each time with 100 ml petroleum ether for 1 minute. Shake the collected petroleum ether extracts once with 25 ml ethanol 75 per cent and add the ethanol to the original alkaline ethanol solution. No further use is made of the petroleum ether extract.

Acidify the combined ethanolic solutions with 15 ml HCl 25 per cent to red with thymol blue as indicator and add 4 ml H_2O if less than 10 g feces have been taken. Extract with 50 ml and 30 ml petroleum ether respectively, by shaking vigorously each time for 1 minute. Combine t petroleum ether extracts and purify by shaking f 1 minute with 40 ml HCl 1 N dissolved in ethan 25 per cent. Repeat this purifying with HCl 1 in ethanol 25 per cent. Then determine for orient tion in 1 ml of the petroleum ether how much fat acid is present, by titrating with 0.1 N ethanol NaOH with thymol blue as indicator to fir change from yellow to green. With a measurin glass pour as much petroleum ether solution corresponds to about 0.5 milliequivalents fatty ac into an Erlenmeyer flask of 200 ml capacity wi ground glass stopper and titrate *exactly* with 0.1 ethanolic NaOH with thymol blue as indicator first change from yellow to green (b ml); durin the titration add so much neutralized ethanol 96 p cent that the titrating liquid quickly mixes with t petroleum ether (5—15 ml). Distill the petroleu ether and ethanol off and dry the soap on a wat bath; *blow off with nitrogen the remaini petroleum ether and ethanol vapors from the sl warm Erlenmeyer flask.* Dissolve the remaini soap, by gentle warming if necessary, in 10 ml 0.2 $KBrO_3$—KBr solution and add 2 ml H_2O. Co add 4 ml CCl_4 and 3 ml HCl 4 N, close the fla immediately and swirl until the fatty acids a completely dissolved in the carbon tetrachlori (about 1 minute). Then quickly add 3 ml KI 1 shake well for 1 minute and titrate with 0.1 N thi sulphate with starch as indicator, repeated shaking well towards the end point of the titrati (*c ml*).

Carry out a blank titration with 10 ml 0.2 $KBrO_3$—KBr (*B ml*).

Calculation

Besides the titration figures obtained, b, c and use is also made of the total number of mi equivalents saturated and unsaturated fatty aci T, excreted per 24 hours, determined by method according to van de Kamer, ten Bokkel Huinink Weijers.

u.f.a. = unsaturated fatty acids
s.f.a. = saturated fatty acids
t.f.a. = unsaturated + saturated fatty ac

milliequivalents bromine bound $= (B-c) \times N_{thio}$

milliequivalents u. f. a. $= (B-c) \times N_{thio} \times 0.5$[1]

m.equiv. u. f. a. per m.equiv. t. f. a. $= \dfrac{(B-c) \times N_{thio} \times 0.5}{b \times N_{ethanol\ NaOH}}$

m.equiv. u. f. a. excreted/24 hrs $= \dfrac{(B-c) \times N_{thio} \times 0.5}{b \times N_{ethanol\ NaOH}} \times T$

mg u. f. a. excreted/24 hrs $= \dfrac{(B-c) \times N_{thio} \times 0.5}{b \times N_{ethanol\ NaOH}} \times T \times 282$[2]

mg t. f. a. excreted/24 hrs $= T \times 270$[2]

mg s. f. a. excreted/24 hrs $= T \times 270 - \dfrac{(B-c) \times N_{thio} \times 0.5}{b \times N_{ethanol\ NaOH}} \times T \times 282$

OBSERVATIONS

1. The first extraction with petroleum ther is made from an alkaline medium, vith the intention of removing nonacid sub-tances which would disturb the determi-ation of the iodine value; this applies specially to the sterols which, in this manner, re removed quantitatively. Since in this peration a small quantity of soap from higher tty acids is also extracted by the petroleum ther, the latter must be shaken once with thanol in order to return these soaps to the riginal ethanolic solution.

2. Extracting twice with petroleum ether om an acid medium which contains about 0 per cent ethanol is sufficient to extract ractically all higher fatty acids (C_{10}, C_{12}, C_{14} etc.) (van de Kamer 1948; van de Kamer, ten Bokkel Huinink & Weijers 1949).

3. Although by far the greatest part of the lower organic acids has remained in the acid ethanol after the extraction with petroleum ether, the latter must still be purified twice with acid ethanol 25 per cent. No higher fatty acids are lost by this extraction, while the lower fatty acids are almost completely removed. The result of the extractions with ethanol of 60 per cent and 25 per cent respectively from petroleum ether is given in Figure 1.

It appears that only the acids with (5), 6 and 8 C-atoms cannot be adequately removed from the petroleum ether. Since these acids can seldom be expected in the feces as products of a fermentation, while, moreover, they only occur in small quantities in a few dietary fats (butter fat, coconut oil and palmseed oil), we may assume that a true picture of the proportion of the saturated and unsaturated higher fatty acids in feces is obtained by titration and the determination of

[1] Multiply by 0.5 because one molecule unturated fatty acid binds 2 atoms of bromine (for olyethenoid fatty acids see discussion).

[2] 282 = average mol. weight of the unsaturated tty acids. 270 = average mol. weight of the total tty acids (van de Kamer 1948).

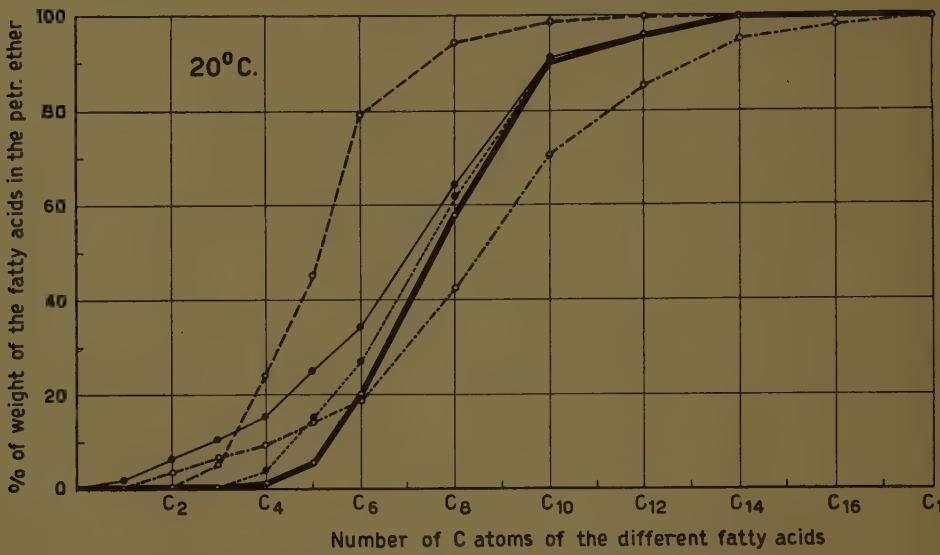

Fig. 1. Partition of the various higher and lower fatty acids after shaking petroleum ether at roo temperature with ethanol 60 per cent, ethanol 25 per cent etc., or vice versa.
The separate solutions were prepared by weighing out a quantity of each fatty acid, dissolving i ethanol, titrating, and after acidifying, making up to an ethanol concentration of 60 and 25 per cen

—.—.—.— 1. shaken once with petroleum ether from ethanol 60 per cent.
———— 2. shaken twice with petroleum ether from ethanol 60 per cent.
— — — — 3. petroleum ether, shaken once with ethanol 25 per cent.
..... 4. shaken twice with petroleum ether from ethanol 60 per cent. petroleum ether then shaken once with ethanol 25 per cent.
▬▬▬ 5. shaken twice with petroleum ether from ethanol 60 per cent. petroleum ether then shaken twice with ethanol 25 per cent.

the iodine value of the fatty acids from the petroleum ether extract, purified as described above.

4. Since both the number of milli-equivalents and the iodine value of the fatty acids must be determined, the iodine used is determined *in the soap* immediately after titration with ethanolic alkali, without first separating the fatty acids. The immediate determination of the iodine value of soap proves to be possible by applying the method of Winkler, after the soap has been dissolved in the solution of $KBrO_3$ and KBr in wat Moreover, the method of Winkler in t form has this advantage, that after minute the bromine has already been quan tatively added, so that the determination be titrated almost at once. The results the following experiments confirm this. fatty acids from the feces of different pers were extracted and purified, their iodi value being determined in various ways

A. According to Winkler, by dissolv the fatty acids in carbon tetrachloride

hen adding $KBrO_3$—KBr solution and HCl. After setting aside for 1 hour in the dark, tration is carried out with 0.1 N thiosulphate after addition of KI solution.

B. According to the above-described modification of Winkler's method, by dissolving the soap in the $KBrO_3$—KBr solution, then cooling and adding CCl_4 and HCl. After swirling for 1 minute it is immediately titrated. For comparison a number of solutions was also set aside in the dark for 5, 10, 15 and 60 minutes before being titrated. The results of these experiments are summarized in Table I.

The determination of the iodine value in the soap thus gives the same results as in the free fatty acids.

5. The total amount of saturated and unsaturated fatty acids excreted per 24 hours is used for the calculation. In practice it proved to be simplest to obtain this amount from a separate determination of the total fatty acid content of the feces (method A according to van de Kamer et al.), determined if desired after purification of the petroleum ether extract with HCl 1 N in ethanol 25 per cent.

In principle, however, it is possible to calculate the total fatty acid content from the titre value *b* of the method of determination described above, provided that the whole determination is carried out quantitatively. This means that a definite portion of the 24 hours' quantity of feces must be weighed out and the petroleum ether extract must be made up to a definite volume, before an aliquot portion is pipetted for the titration of the fatty acids and the determination of the iodine value.

Table I. *Determination of the iodine value according to Winkler and according to a modified method of Winkler, in the fatty acids from the feces of various patients and in the fatty acids of a few fats.*

Fatty acids	Winkler method	Modified Winkler method					
		Interaction time with bromine in minutes					
		1/4	1	5	10	15	60
Feces I	26, 25, 27, 26	-	25, 26	-	-	-	-
			26, 27	-	-	-	-
» II	27, 27	-	27, 27	-	-	-	-
» III	23	-	23, 23	-	-	-	-
» IV	37, 38, 37, 38	-	35, 36	-	-	-	-
			37, 36	-	-	-	-
» V	76, 77	-	78	-	-	-	-
» VI	-	20	-	22	-	20,21	
» VII	-	33,32	32, 32	-	33,33	-	-
Butter fat	40, 40	-	40, 41	-	-	-	-
			39, 40				
Palm oil	59	-	61	-	-	-	-
Peanut oil	88	-	88	-	-	-	-

Finally, it must be pointed out that in the calculation the possible presence of polyethenoid fatty acids (linoleic acid, linolenic acid etc.) has not been taken into account. Moreover, in the case of healthy persons, as well as of patients suffering from steatorrhea, the excretion of unsaturated fatty acids is very small, as will be shown in subsequent articles (Weijers et al., 1953) so that the polyethenoid fatty acids can be neglected. It appeared to us namely, through determination of the thiocyanogen and iodine value in the purified fatty acids from feces of seven different patients and from a number of mixed samples, that only 10—25 per cent of the unsaturated fatty acids consist of linoleic acid.

Addendum

Recently we also made use of the method of Wijs for the determination of the iodine value, by dissolving the soaps in 20 ml 0.2 N Wijs-solution, followed by titration after 60 minutes. This method gives the same results.

SUMMARY

A method is described for the quantitative determination of the saturated and unsaturated higher fatty acids in feces. The method is based on the titration of the fatty acids in a purified petroleum ether extract of feces, followed by the determination of the bromine binding capacity of the soap formed.

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BREAKDOWN OF DEXTRAN BY HUMAN FECES

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In 1950, Engstrand & Åberg studied the te of intravenously infused dextran in the ody. They found that a part of the dextran fused was excreted in the gastrointestinal act. In the feces from patients who had ceived dextran, no dextran could be shown. When fresh human feces was mixed with extran, there was a rapid decrease in the extran content of the mixture. Engstrand Åberg presumed this to be due to bacterial tion.

Hehre & Sery (1952) have confirmed that testinal bacteria are able to break down extran. These bacteria were found to be naerobic and, according to these authors, part of the split products was some reucing sugar or sugars.

In order to try to isolate the split procts of dextran, broken down by the action intestinal bacteria, the following experients have been performed.

Fresh feces from 10 normal subjects were ixed and diluted with 4 parts of physiogical saline. The mixture was centrifuged nd the clear supernatant fluid was decanted nd kept cold.

Ten ml of a 6 per cent dextran solution, Macrodex", was mixed with 10 ml of different phosphate buffer solutions, the pH nging from 5.29 to 8.00. To this mixture, equal amount by volume of the feces pernatant was added and the mixture was incubated for 3 days at 37° C. The same procedure was performed in another series using physiological saline instead of dextran. The content of reducing substance was estimated before and after the incubation with the Somogyi method (1937). The carbohydrate content of the incubates was estimated with the carbazol reaction according to Dische as modified by Gurin and Hood (1939).

An 82 per cent decrease in the carbohydrate content of the dextran-incubate occurred, at a pH of 6.24, when measured by the carbazol reaction. At other pH values the decrease was considerably lower. There was no increase in reducing substance. The reference incubate, which contained saline instead of dextran, showed a constant amount of carbohydrates and reducing substances during the incubation. When the same experiment was performed with boiled feces and dextran, there was no decrease in the carbohydrate content.

It thus seemed probable that fresh human feces is able to break down dextran. The optimum pH was 6.24 and the split products were not reducing substances.

It was possible that the split products were organic acids, and therefore the following experiment was performed.

A dilution of 1 part of human feces in 4 parts of phosphate buffer solution at pH 6.24, was mixed with "Macrodex", so that the

final concentration of carbohydrates was 2.85 mg per ml, when estimated as dextran with the carbazol reaction. Another part of the same feces-phosphate buffer solution was mixed with physiological saline. The final volume of both mixtures was 1600 ml respectively. The mixtures were incubated at 37° C for 3 days.

The carbohydrate content estimated with the carbazol reaction decreased during the 3 days from 2.85 mg per ml to 0.735 mg per ml in the dextran-containing incubate, whereas the dextran-free incubate showed a decrease from 0.510 to 0.380 mg per ml. In both incubates, there was no increase in reducing substances estimated according to Somogyi (1937). To both incubates 150 ml of 30 per cent (w/v) of sulphuric acid was added and the incubates were each distilled at 100° C until 1000 ml had distilled off. The distillates, which were acid, were then electrometrically titrated with 0.1 N sodium hydroxide. It was found that the distillate from the mixture containing dextran required 24.2 ml of the sodium hydroxide solution per 100 ml, while the other distillate required 4.3 ml.

On incubation, a considerable amount of volatile organic acid was thus formed, viz. approximately 1.45 g, if calculated as acetic acid, from 3.38 g carbohydrate.

The distillation constants according Clark (1943) of the two distillates were d termined. It was found that acetic acid ma up the main part of both distillates.

SUMMARY

When dextran is mixed with human fe in a phosphate buffer solution at pH 6.2 a rapid break down of the carbohydra occurs. There is no increase in reducing st stances. However, an increase in volat acids, mainly acetic acid occurs.

ACKNOWLEDGEMENT

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TYPING OF HEMOLYTIC STREPTOCOCCI IN SCARLET FEVER AND OTHER STREPTOCOCCAL DISEASES

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(Received for publication July 8, 1952)

During the years 1947 to 1949 there was high incidence of scarlet fever in Stockolm. The material from this epidemic has een investigated from different aspects by ie following: Strøm (1948, 1950), Bengtson, irke and Wingstrand (1951), Lagercrantz 1950), Levander (1952). Our paper is conerned purely with the bacteriological aspect f the disease.

MATERIAL

The material studied consists of 1807 cases of arlet fever occurring in Stockholm between the rst of April 1947 and the end of May 1949. This aterial covers a representativ number of all the ses of scarlet fever which occurred in Stockholm this time. In addition another 382 cases of other fectious diseases caused by the hemolytic streptoccus were studied. The patients with scarlet fever ere isolated in the hospital and most of them were eated with penicillin by different methods (Strøm 948, 1950). Complicated cases are those showing ie or more of the following conditions: synovitis, yocarditis, nephritis, otitis media, sinusitis, tonlitis, lymphadenitis, impetigo contagiosa and ronychia.

METHODS

Nose and throat swabs were taken from every tient on admission, and daily for the next three ys, and this was followed by weekly swabs, and ally before the patient was discharged swabs ere taken on three consecutive days. In all comicated cases additional swabs were taken, when cessary. All swabs were planted out on horse blood agar plates, and the colonies were tested for the presence of soluble streptolysin before typing. Griffith's slide agglutination technique for typing was used throughout these series. Only samples which gave typical hemolytic colonies were investigated further for typing and so on.

RESULTS

As shown in Fig. 1, a high percentage of hemolytic streptococci were typed in the scarlet fever cases and we found that these belonged to several different types. As shown in Table I there was one type which predominated at different periods. As an example the Table shows that during the period April—July 1947 type 6 was predominant and this type was followed by type 5 during the period Aug.—Nov. Type 6 was also the most common amongst the other streptococcal cases (tonsillitis etc.). (See Table II.)

The most common streptococcal infection other than scarlet fever was tonsillitis. The age distribution of the patients with scarlet fever and tonsillitis is shown in Fig. 2.

The cases treated with penicillin showed few recurrences of hemolytic streptococci and complications were uncommon. It was only possible in 122 uncomplicated and 118 complicated cases to type the organisms on more than one occasion (Fig. 3). In this group of cases it was found that the organisms mainly

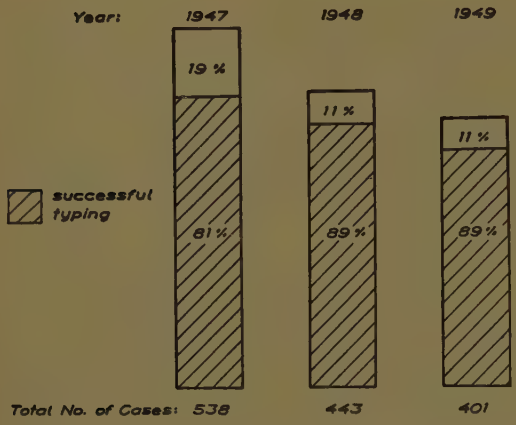

Fig. 1. Shows the percentage of organisms typed.

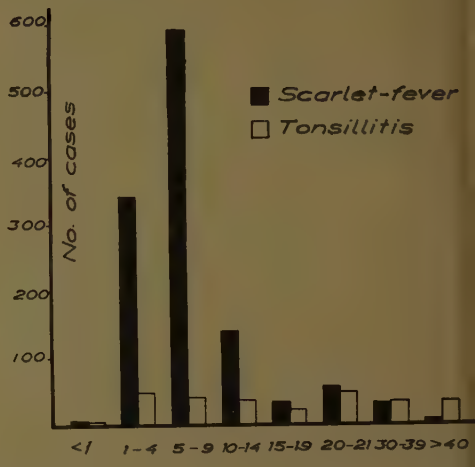

Fig. 2. To show the age distribution of cases scarlet-fever and tonsillitis.

belonged to the same type. A change of type was present in 17 per cent of complicated cases but only in 7 per cent of uncomplicated. The difference in these percentages is significant.

Table I. *To show the seasonal incidence of predominating types. The figures within brackets represent the next most common type.*

	Predominating type		
	1947	1948	1949
January		28	4
February		28	4
March		28	4
April	6	6	4
May	6	6	4
June	6	28	
July	6	28	
August	5(6)	22(28)	
September	5	6	
October	5	6	
November	5	27(6)	
December	5(28)	4	

In 10 relapsing cases of scarlet fever was possible to type the organism on b occasions. Eight of these showed differ type and two the same type.

As relapsing cases of scarlet fever due the same type of organism are uncomm we thought it would be worth while to rec two such cases:

No. 1. (No. 1448/49.) The patient was a th year old boy, whose twin brother was also trea for scarlet fever. The patient was admitted hospital on Feb. 26th 1949, and received 75 units of procaine pinicillin daily for the first days. At the end of four days the fever had s sided. Nose and throat swabs revealed hemol streptococci group A type 4. There were no c plications. The patient was discharged from ho tal on March 18th 1949, free from hemol streptococci and six days later developed a typ scarlet fever rash and was readmitted to hosp on March 24th. On admission there was a typ scarlatina rash, which gave a positive Sch Charlton's reaction. He was again treated v procaine penicillin (100.000 units daily fo days). Throat and nasal swabs revealed hemol streptococci group A type 4. There were no c plications.

able II. *A comparison of the frequency dominating types present. Cases of scarlet fever and other streptococcal conditions.*

Year	Type	Number of cases	
		Scarlatina	Other streptococcal conditions
	5	78	18
	6	49	15
47	2	44	11
	29	23	6
	42	15	8
	28	17	5
	6	82	30
	28	72	36
48	44	52	11
	26	49	10
	27	36	12
	11	17	11

No. 2. A seven year old boy was admitted on ct. 12th 1948 and was treated for a typical arlet fever with procaine penicillin (50.000 units ree or four times daily orally for six days). Nose d throat swabs revealed hemolytic streptococci oup A type 6. There were no complications and was discharged from hospital on Nov. 3rd, free om streptococci. Six days later he was readitted to the hospital with a typical scarlet ver. Nose and throat swabs produced hemolytic reptococci group A type 6. In order to obtain more natural immunity penicillin treatment was layed until Nov. 26th (penicillin procaine 100.000 its per day for 4 days). There were no comications and he was discharged on Nov. 29th.

We were fortunate to have the opportunity investigate several small outbreaks in milies, schools, day-nurseries etc. These sults are shown in Fig. 4. As a rule only vo members of the collectives were involved an outbreak (1947 there are 42 outbreaks mprising 87 cases). Usually the individus in an outbreak had the same type of strepcocci, but sometimes different clinical contions e. g. one case showed scarlatinal rash, nother tonsillitis etc.

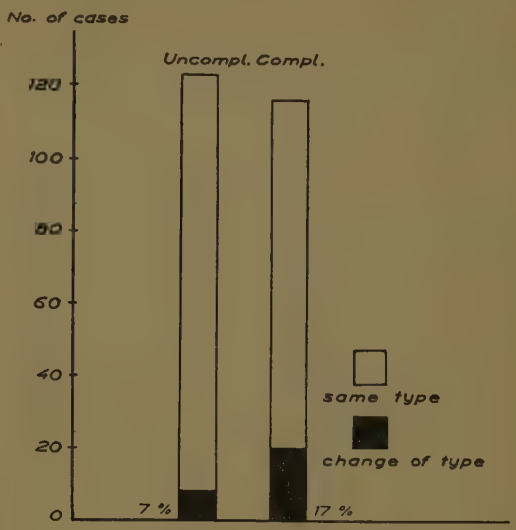

Fig. 3. Showing a comparison of the percentage of change of type in complicated and uncomplicated cases, in which the typing was performed on more than one occasion.

DISCUSSION

In Griffith's slide-agglutination-technique (1934) the streptococci are typed mainly according to their T-antigen. As the T-antigen is common to several different types this method of typing is not specific. A more thorough method of typing is that described by R. Lancefield (1943) based on the presence of the M-antigen. Griffith's technique was used in these series. In our opinion a significant change of type can be demonstrated with Griffith's technique only if the streptococci typed contain different T-antigens. Previous workers on this subject have not considered this fact. We were not able to find such a high frequency of type-change in our material as previous workers (Ehinger 1945, Ernst 1942). This is probably at first hand due to the method of treatment (isolation of the patients and penicillin treatment)

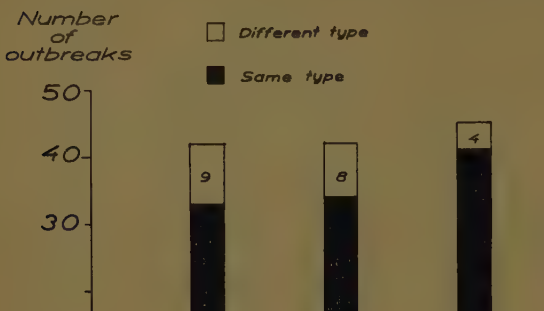

Fig. 4. Showing the number of localized outbreaks, in which typing was performed. Individuals in most of the outbreaks showed the same type.

which reduced the risk of cross-infection. But it might also depend on the method adopted for the confirmation of change in type.

Amongst the complicated cases cross infection by different types was not common but in the patients who developed complications the incidence of cross infections was significantly higher. On the whole a change of type did not seem to be of any great importance in the development of complications in this material. This fact must in the first place be due to the penicillin treatment.

Since the penicillin treatment for scarlet fever began there has been an increased number of relapsing cases of scarlet fever in the Hospital for Contagious Diseases in Stockholm (see also Strøm 1948). Unfortunately we have not been able to type the streptococci in relapsing cases on both occasions except in twelve cases. The two cases that showed the same type on both occasions are specially interesting as the immunity is believed to type specific. T. Jersild (1948) was the fi to publish a case of relapsing scarlet fe due to the same type, group A type 4 A.

One knows that the early penicillin tre ment effectively kills the streptococci a thus the body does not have the opportun to form an immunity against the tox Ström (1948) and Lagercrantz (1950) ha shown in this material that a rise in an streptolysin titre is inhibited with early a effective penicillin therapy. Before dischar from the hospital patients are transformed a "clean ward" i. e. all patients have negat nose and throat swabs. Thus it may assumed that reinfection does not take pl in the hospital. It would appear that child is reinfected at home either by parer brothers or sisters who are streptococcal c riers, or possibly by a dust-borne organis

We succeeded in typing a fairly high p centage of our cases, and this was proba due to the fact that we were using fres isolated strains and that a few types w predominant in this community.

As Ernst (1942), Ehinger (1945) and oth have shown, there are no specific types scarlet fever. The types which are predo nant in scarlet fever are predominant also other streptococcal diseases (Table II). T rash in scarlet fever is probably due to hyp sensitivity to the Dick toxin. The age dis bution of our material (Fig. 2) correspo with previous workers, Powers and Boisv (1944). Scarlet fever is most common the age period in which allergic skin re tions mainly occur i. e. 3—11 years. La the patient is more prone to get tonsil etc. In our investigations during locali

tbreaks we were able to show that in some ses a patient may develop scarlatina, whilst hers developed tonsillitis. This was usually used by the same type (see Fig. 4).

SUMMARY

1. During the period 1947 to 1949 we vestigated 1807 cases of scarlet fever as ell as 382 cases of other streptococcal con-tions caused by the hemolytic streptococcus. he Griffith's slide agglutination technique as used.

2. About 80 per cent of the cases were ped. Although a great many types were und, only the types with different T-antigen ere considered as separate entities.

3. A change of type, i. e. a change of types ith different T-antigen, was found signifi-ntly more often among the complicated an the uncomplicated cases of scarlet fever. ost cases had their complications without change of type. In twelve cases of relapsing arlet fever the organism was typed on both casions. Ten showed different types and vo had the same types — this must be due the penicillin-treatment.

4. During the investigation period a few pes were predominant among the cases of arlet fever as well as in the cases of other reptococcal diseases. Considering the types streptococci, there is no etiological dif-rence between scarlet fever and other strep-coccal diseases.

5. Scarlet fever was most common in chil-en between 3—11 years of age, whereas nsillitis was just as common in adults as children.

6. The individuals in small outbreaks usually showed the same type of streptococci, but the clinical conditions differed sometimes.

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AN AGGLUTINOGEN SKIN TEST IN PERTUSSIS

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During the last two decades several attempts have been made to secure a satisfactory skin test for pertussis. Mostly these tests have been used to appraise the efficacy of pertussis vaccination, but few have been performed during and after attacks of pertussis. Opinions as to the results have been divergent (Smolens & Mudd 1943, 1946, Felton 1946, Miller et al. 1948, Felton, Flosdorf 1946, Halpern & Halpern 1948, Keller et al. 1942 and Kohn et al. 1950). Some animal experiments have shown good correlation between the cutaneous response and the serologic reactions (Halpern & Halpern 1948).

MATERIAL

In connection with our investigation (1950—1951) on the value of chloromycetin in pertussis (Hedlund & Mossberg 1951) we have used an agglutinogen prepared from *Haemophilus pertussis* for skin tests. The material consists of pertussis cases in children of all ages (112 cases), but very few (16 cases) less than one year of age. The chloromycetin-treated children were divided into preparoxysmal and paroxysmal cases. The untreated cases were not divided in this way. The skin test was performed at different stages of the disease, and during hospitalization it was repeated at least every other week (Group 2).

Apart from this group of children treated pertussis in the hospital, the skin test was a performed in other children. One group cons of children (61 cases) without any known cont with *Haemophilus pertussis,* viz. neither as di nosed disease nor through vaccination (Group

Two other groups comprise children (15 cas vaccinated during the years 1947—1950 (Group and those (51 cases) who have had pertussis dur the years 1941—1950 (Group 4).

METHODS

The agglutinogen for the skin test has b prepared according to Smolens & Mudd (19 with the following modifications.

Hemophilus pertussis in Phase I was grown a casein hydrolysate medium (Cohen, Wheeler) 48 hours. The culture was passed through a Laval separator and the bacteria washed two three times with distilled water. The cells w extracted with 0.1 n hydrochloric acid pH 1.8— during 24 hours at 56° C.

NaOH (N/1) was added slowly to pH 4.0. resulting precipitate was washed three times w distilled water. The washings were added to supernatant fluid. NaOH was again added to 6.0 and to pH 7.0. At both these pH there occur a precipitation. Both precipitates were was with distilled water and the washings were ad to the supernatant fluid.

To the extract was added an equal volume saturated ammonium sulfate. The precipitate washed twice with 50 per cent ammonium sul solution.

The precipitate was taken up in a volume water equal to one-half the volume of the extr present. This suspension was dialyzed agai

[1] The clinical experiments have been carried out by Dr. Mossberg.

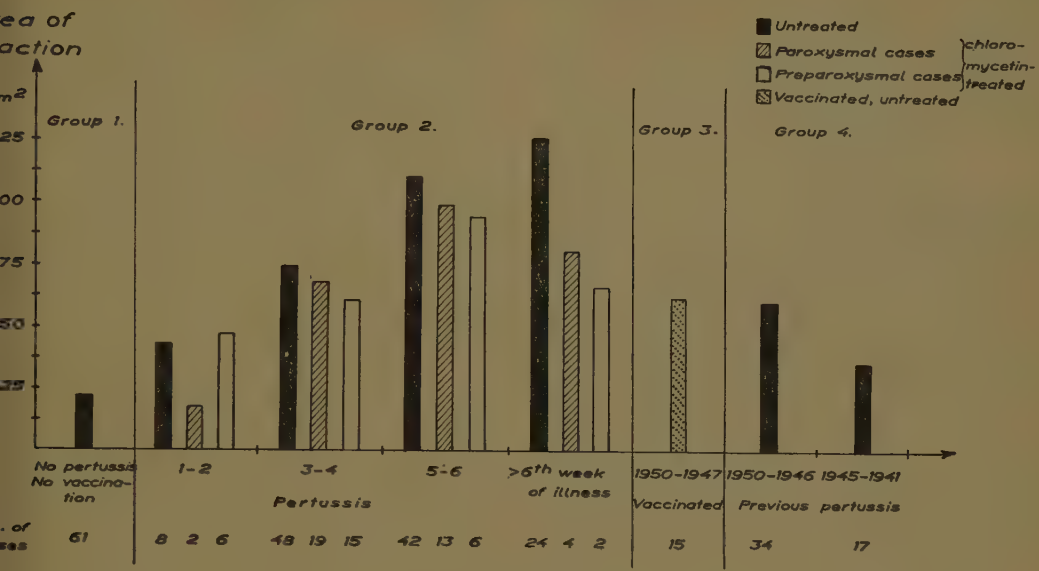

Fig. 1.

ning tap water for 3—4 hours and running illed water for 24 hours. The dialyzed material centrifuged and insoluble particles were re-ved. To the clear dialyzed material was added -half volume of saturated picric acid. The ture was allowed to stand for two to three rs in a refrigerator and then centrifuged. The cipitate was taken up in about the same volume vater used for the ammonium sulfate precipitate. suspension was then dialyzed against running water until the last trace of yellow color had ppeared, which took about two weeks. After-rds centrifugation and removal of insoluble erial.

The agglutinogen, dissolved 1:10 in normal ne, was injected intracutaneously in the volar face of the arm. The reaction culminated after ours when the flare was measured. The largest neter and that at right angles to it were recorded illimeters. As the flare in shape most frequently mbled an ellipse, the surface formula for this re was used in calculating the size of the flare. eals were very frequent but somewhat irregular often technically difficult to measure. For this son the wheal was not recorded.

RESULTS

The results are presented in Figure 1. The children suffering from pertussis (112 cases) are grouped according to the stage of the disease (Group 2). Both chloromycetin treated (41 cases) and untreated (71 cases) children at the beginnig of the disease showed small reactions, but the reactions were somewhat larger than in children without any known contact with *Haemophilus pertussis* (Group 1). During the course of disease the size of the reaction increased and culminated after more than 6 weeks, when recovery usually begins. As regards this development, there is no difference between the treated and untreated children, but those treated with chloromycetin had lower means than the untreated ones, and a tendency towards the lowest rates was noted in the cases treated in the preparoxysmal stage of the disease. The increase is statistically valid in

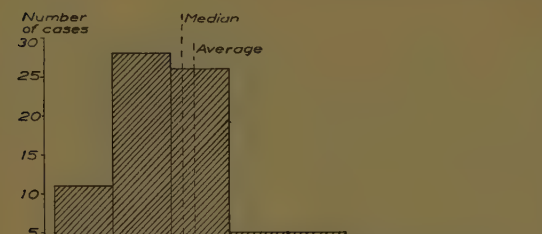

Fig. 2. Distribution of cases in group 1 (Fig. 1).

both treated and untreated children, but within the different stages of the disease the material is too small to yield statistical validity as to treated versus untreated children.

The number of skin tests performed on every child in Group 2 was on an average 1.7. It should also be mentioned that there were no large variations in the size of the flare within the different stages of the disease in Group 2. Nor did tests repeated after a fortnight in 8 cases in Group 1 entail increasing size of the skin reaction.

Figure 2 shows the distribution of cases in Group 1. The average area of reaction within this group was 24 mm². The distribution of cases within the group was oblique with the median at an area of reaction of about 22 mm². The other groups had a more regular distribution.

Children who had had pertussis earlier (Group 4) exhibited a reaction smaller in size than children who had just recovered from the disease(Group 2). The figure shows that children with pertussis 1941—1945 had smaller reactions (39.8 ± 6.8 mm²; $n = 17$), than those who were ill 1946—1950 (63.4 ± 4.8 mm²; $n = 34$), but still larger than those without any known contact with *Haemophilus pertussis* (Group 1) (± 2.2 mm²; $n = 61$). Statistically the ferences between these three groups highly significant.

The size of the skin reaction in the c dren vaccinated during the years 1947—1 (Group 3) (66.7 ± 16.2; $n = 15$) was c parable with that of the skin reaction in children who had had the disease during years 1946—1950.

DISCUSSION

The value of skin tests for pertussis man has been disputed. Many investiga believe these skin reactions to be non-spec Judging by our results which show creasing sensitiveness to the agglutino during the course of disease (Group 2), reaction seems to be due to the pertus Obviously, this sensitiveness may also re from vaccination (Group 3). Many y after the illness had subsided, the pati still responded to the agglutinogen (Gr 4). After five to ten years the reaction still twice as large as in the children wit any history of pertussis (Group 1). M of the children in this Group 1, however, sponded to the agglutinogen (Fig. 2).. is probably to be explained by the fact the disease had been overlooked or had ta a subclinical course in these cases.

As is evident from Fig. 2, most of children who had not had any contact pertussis exhibited an area of reaction of than 30 mm² corresponding to a diamete 6 mm. Therefore one would venture to that a reaction area of more than 40 corresponding to a diameter of about 7 argues in favour of an infection with *Hae philus pertussis.*

Treatment with antibiotics at an early ge of a bacterial disease seems to result an immunity which is weaker than that quired without treatment. One of our asons for using the skin test was to collect idence on this point. This question, how-er, cannot be decided on the basis of the esent series.

On the other hand, the investigation shows at there is a rise in reactivity during the urse of disease. It also seems that the ated cases show fewer responses than the treated ones, but here statistical validity lacking. Furthermore, in those cases here chloromycetin treatment was insti-ed during the preparoxysmal stage of the sease, there was a tendency towards milder in reactions as compared with those treated the paroxysmal stage of the disease.

However, supposing that the size of the in reaction indicates the state of immunity which is not proved — the present in-stigation yields information about the velopment of immunity during, and sub-lence after, an attack of pertussis. The in test perhaps also provides information to whether or not a child is susceptible pertussis.

SUMMARY

An agglutinogen skin test was performed 112 children suffering from pertussis, in children with a history of pertussis, in children without any known contact with rtussis and in 15 children vaccinated ainst pertussis.

The agglutinogen was prepared according Smolens & Mudd with some modifications. During the attack of pertussis the size of skin response increased and culminated after the sixth week of illness. Children treated with chloromycetin had lower means for the skin reaction than the untreated ones, with a tendency towards the lowest rates in the preparoxysmal cases.

Children with a history of pertussis showed decreasing size of the responses to the agglutinogen, with smaller reactions when more time had passed since the illness. Five to ten years afterwards the skin reaction was still significantly larger than in children without any known contact with *Haemophilus pertussis*.

Children vaccinated against pertussis during the preceding three years presented a cutaneous response comparable with those who had had the disease during the last five years.

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THE DETERMINATION OF ENDOGENOUS »CREATININ IN PLASMA AND URINE

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Numerous publications concerning plasma endogenous "creatinine" and endogenous "creatinine" clearance have appeared during recent years. Although endogenous "creatinine" determinations seem comparatively simple, the figures published for both plasma "creatinine" and its clearance differ considerably. Behre & Benedict's (1922) statement that "each investigator, using a new technique, and one seemingly accurate, is able to get figures quite different from those obtained by any previous method" seems to apply not only to creatine, but to creatinine as well.

Table I shows the results of some recent studies of endogenous "creatinine" and the methods employed. Brod & Sirota (1948) reported an average value of 0.91 mg/100 ml, while Brod & Kotátko (1949) using exactly the same technique, found 1.03 mg/100 ml. The average value obtained by Barrett & Addis (1947) was 1.02 mg/100 ml. Also the range differs greatly. The upper limit of the normal range is of particular importance as far as the diagnostic value of the test is concerned. Brod & Sirota found 1.10 mg/100 ml as the upper normal limit, while Brod & Kotátko reported it to as much as 1.56 mg. In a normal series investigated by Blegen, Haugen & Aas (1949) the highest value was 1.25 mg/100 ml, which accords with that reported by Barrett & Addis (the values in their normal material ranged from 0.7 1.26 mg/100 ml in 95 per cent of the cas

These investigators all used the same a for precipitation of the plasma protein (J rett & Addis used serum instead of plas Blegen et al. in some instances used a So gyi filtrate). While Blegen et al. use saturated solution of picric acid for the J reaction, Barrett & Addis used a wea picric acid solution and made allowa for the temperature. Brod & Sirota Brod & Kotátko preferred the Bonsne Taussky modification (1945) of the J method. All these investigations made of modern colorimeters. Blegen et al. the color after 15 minutes, Barrett & A after 10 minutes, and Brod & Kotátko 15 minutes, while Brod & Sirota gave details concerning the reading time.

It is possible that the differences in no figures and upper normal limits are ca by differences in methods. For that rea I have examined what role different pre precipitants play in the determination endogenous "creatinine". The influenc the temperature of the solutions at the co metration and the influence of time on c development have also been examined. ther, various factors concerning the stances called "pseudocreatinine", i. e. creatinine plasma constituents giving Jaffé reaction, are discussed.

Table I.

Authors	Method	Endogenous "creatinine" values
arrett & Addis 1947 ..	"Standard Folin Wu method" Picric acid 11.75 g/1000 ml All reagents in water bath (24.5°—26.2°) Color read after 10 min.	Average 1.02 mg/100 ml 95 per cent of normals within 0.78—1.26 mg/100 ml
rod & Sirota 1948	Own modification of Folin & Wu precipitation (1 volume of plasma, 1 volume of water, 1 volume of 5 per cent sodium tungstate, 1 volume of 0.66 N H_2SO_4) Bonsnes & Taussky's modification of the Jaffé reaction	Average 0.91 mg/100 ml (0.64—1.10 mg/100 ml)
rod & Kotátko 1949 ..	Same method as that used by by Brod & Sirota. Color read after 15 min.	Average 1.03 mg/100 ml (0.64—1.56 mg/100 ml)
legen, Haugen & Aas 1949	1) Folin & Wu precipitation (dilution 1 : 5) 2) Somogyi precipitation Saturated picric acid. Color read after 15 min.	0.74—1.25 mg/100 ml

METHODS

Heparinized plasma from normal subjects, i. e. bjects with normal blood pressure and normal ine, was used, unless otherwise stated.

As deproteinizing agent various concentrations sodium tungstate and sulfuric acid were used:

1) The original Folin & Wu method (1919), ing plasma instead of blood. To one volume of asma are added 7 volumes of water and one volume ch of a 10 per cent sodium tungstate solution and o-thirds N sulfuric acid. Barrett & Addis used e same protein precipitation method.

2) The Folin & Wu method, using a 1 : 5 plasma lution. To one volume of plasma are added 2 lumes of water and one volume each of a 10 per nt sodium tungstate solution and two-thirds sulfuric acid. This method was used also by legen et al.

3) Wu's modification (1922) of the Folin & u method. To one volume of plasma are added volumes of water and one-half volume each of 10 per cent sodium tungstate solution and two-irds N sulfuric acid.

4) Brod & Sirota's modification (1948) of the Folin & Wu method. To one volume of plasma are added one volume of water and one volume each of a 5 per cent solution of sodium tungstate and 0.66 N sulfuric acid. This method was also used by Brod & Kotátko.

For the Jaffé reaction use was made of saturated picric acid and a 10 per cent solution of sodium hydroxide. In the study of the influence of temperature upon the color, use was also made of Peters' (1942) weaker picric acid (11.75 g/1000 ml) and Bonsnes & Taussky's (1945) modification of the Jaffé reaction, i. e. 3 volumes of the unknown, one volume each of a 0.04 M picric acid and 0.75 N sodium hyroxide solution.

The color was read in a Coleman Junior spectrophotometer at a wavelength of 500 mμ.

The influence of the temperature.

It is an established fact that the temperature affects the colorimetric determination of creatinine. Peters (1942) stated that he

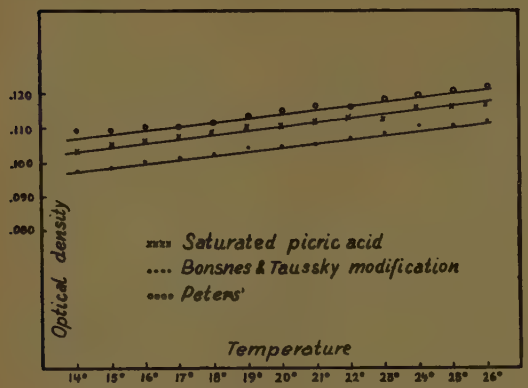

Fig.1.

did not succeed in obtaining identical calibration curves at summer and winter temperature, a circumstance which has been considered caused by a precipitation of the highly saturated picric acid at lower temperature. Peters therefore adopted the use of a somewhat weaker picric acid solution (11.75 g/1000 ml) which is said "just sufficiently unsaturated" to exclude the influence of the temperature.

Figure 1 shows the influence of the temperature on the color of aqueous creatinine solutions in the Jaffé reaction. It will be seen that the optical density increased with increasing temperature, not only when saturated picric acid solution was used, but also with the weaker solution of Peters. The same applies to the Bonsnes & Taussky modification. The essential point is that all solutions, both for plotting the calibration curve and for reading of the unknowns, are kept at the same temperature. For this a water bath must be used. The actual temperature is of little importance, although a temperature close to room temperature is preferable. An essential point is that the temperature of the solutions is not allow to rise at the colorimetration.

Lauson (1951) has drawn attention to t fact that "the water-alkaline picrate bla darkens slightly when repeatedly expos to the light beam passing through the cuve —". This has also been my experience. T darkening is due to a rise in the temperatu of the solution. If the temperature of t blank is kept constant during the colorimet tion, there will be no darkening. The bla should be replaced in the water bath betwe each reading.

The influence of the time factor

General agreement exists that the co development of the Jaffé reaction in aque solutions of creatinine reaches its maxim within a short time after the addition of alkaline picrate. According to Hunter Campbell (1917) the maximum color dev opment occurs within 10 minutes. This confirmed by Hayman, Johnston & Ben

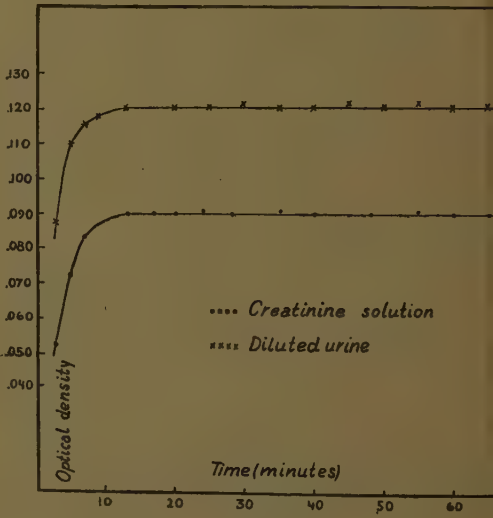

Fig. 2.

1935). Bonsnes & Taussky (1945) stated at maximum color development in their odification of the Jaffé reaction occurred ter 15 minutes, and that the color remained nstant for at least 30 minutes thereafter. igure 2 shows that this applies also to dilu- ons of normal urine. Aqueous dilutions of eatinine and dilutions of normal urine both owed a marked increase of the color in- nsity during the first minutes following the ldition of alkaline picrate, while no further crease was observed after 10 minutes.

Opinions differ, on the other hand, con- rning the influence of the time factor on e color development in plasma filtrates. unter & Campbell found an increase of the lor intensity of picric acid plasma filtrates ter 10 minutes. The same observation was ade by Hayman, Johnston & Bender in ichloracetic acid filtrates. Bonsnes & aussky, in their modification of the Jaffé eaction, were unable to demonstrate any crease in color intensity during the sub-

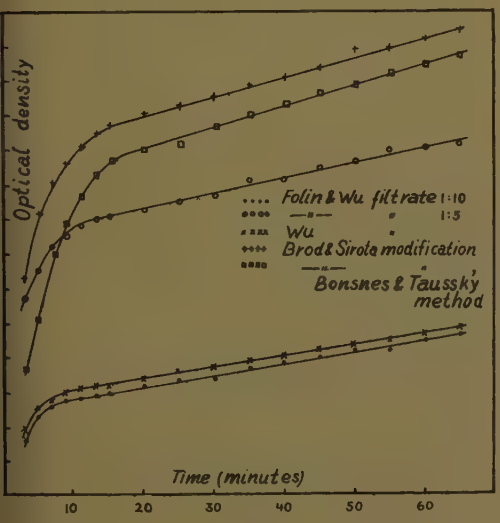

Fig. 3.

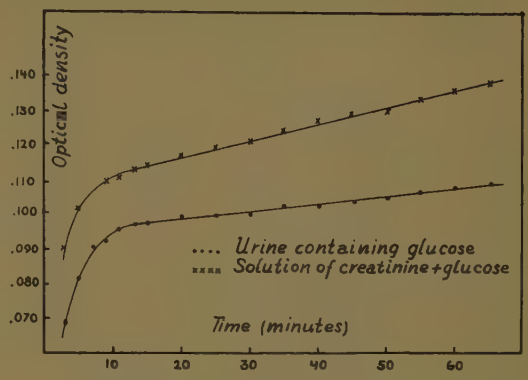

Fig. 4.

sequent 30 minutes following maximum color development, although they admitted that a darkening may occur after 40 minutes. Hare (1950) studied the color development in $CdSO_4$, Somogyi, iron, and Folin & Wu filtrates, all in 1 : 5 dilutions, and found a darkening of the color in all these filtrates. For this reason she considered them all unsuitable for plasma creatinine determinations. Bucht (1951), on the other hand, found no darkening of the color in Folin & Wu filtrates after 15 minutes.

The possibility exists that the concentration of sodium tungstate or sulfuric acid may influence the color development in Folin & Wu filtrates. The influence of the time factor on the Jaffé reaction in the four types of plasma filtrates described was therefore investigated.

Figure 3 shows that an even increase of the color intensity was noted in all filtrates after 10 minutes. This confirms the observations published by Hare. It must be presumed that the darkening after 10 minutes is not caused by the deproteinizing agent, but by substances contained in the plasma.

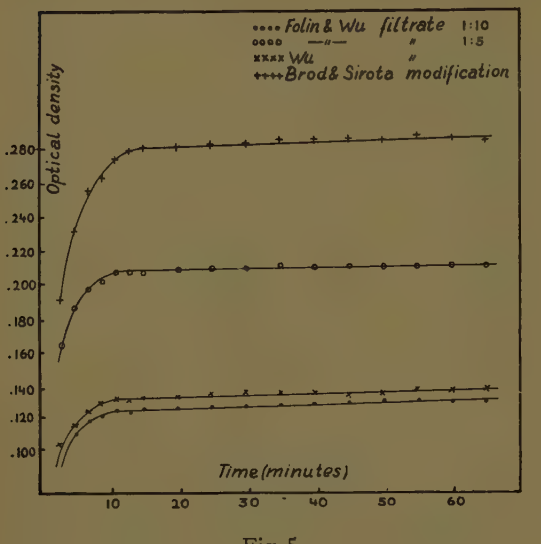

Fig. 5.

Figure 3 shows that the same increase in optical density occurred also when the Bonsnes & Taussky modification was used in a Brod & Sirota filtrate. Exactly the same result was obtained, using the other different tungstic acid filtrates (not plotted in the table). This is not in agreement with the results obtained by Bonsnes & Taussky.

Bucht, as mentioned, was not able to confirm the findings reported by Hare. He considered impurities of the deproteinizing agents the reason for Hare's results, and stated that traces of acetone, for instance, might give increasing color intensity after 15 minutes. All reagents used in the present studies, however, were of first grade quality, and interference by impurities can be excluded. The influence of time was also separately studied in aqueous creatinine solutions which were treated as plasma (addition of sodium tungstate, sulfuric acid, etc.). In these experiments no increase in optical density was observed after 10 minutes, this be taken as additional proof of the purity of deproteinizing agents.

The substances contained in plasma whi besides cretanine, may contribute to characteristic color development with al line picrate are not fully known. Hunter Campbell held the opinion that glucose "the only substance in plasma capable simulating the reaction for creatinine". Fr Figure 4 it is seen that aqueous solutions creatinine + glucose showed an even crease in optical density. From the sa figure it will be seen that a similar darken of diluted urine containing glucose (fro diabetic without ketonuria occurred af 10 minutes. Thus it seems justified to assu that the glucose contributes to the darken also in plasma filtrates, after 10 minutes

Glucolysis occurs in plasma on standi and this fact was utilized in a study of influence of the glucose on the color de opment after 10 minutes. Plasma sam were allowed to stand until a reduction 20 mg/100 ml (by the Hagedorn-Nor Jensen method) had occurred. From Fig 5 it will be seen that only a slight incre in optical density occurred after 10 minu The slight darkening observed is prob not due to glucose, but to other substan as the 20 mg per cent reduction most li was due to something else. The Haged Norman Jensen method is not specific glucose.

It may be objected that also these r glucose substances may be destroyed standing. Glucose was therefore added glucolysed plasma in a concentration of mg/100 ml. The influence of the time fa in filtrates of this plasma is shown in Fi

Table II. *Plasma "creatinine" values by four different tungstic acid precipitation methods.*

	Wu precipitation	Folin & Wu (1:10)	Folin & Wu (1:5)	Brod & Sirota modification
	1.01	0.90	0.89	0.90
	1.09	1.02	0.89	0.95
	1.02	0.91	0.89	0.90
	1.03	1.01	0.90	0.90
	0.95	0.94	0.81	0.91
	0.88	0.88	0.77	0.79
	1.03	0.96	0.90	0.96
	1.02	1.02	0.93	0.96
	1.18	1.05	0.98	1.03
	0.98	0.88	0.82	0.86
	0.99	0.96	0.88	0.95
	1.06	1.02	0.92	0.96
	1.17	1.09	1.05	1.08
	0.85	0.81	0.78	0.76
	0.77	0.76	0.74	0.77
	0.99	0.96	0.88	0.95
	1.18	1.05	0.98	1.03
	1.02	0.92	0.88	0.86
	1.04	0.94	0.88	0.79
	0.89	0.85	0.81	0.77
Average	1.01	0.95	0.88	0.91

. It will be seen that a darkening occurred fter 10 minutes and that this is more narked in Figure 6 than in Figure 5. This roves that the glucose is essential for the olor development after 10 minutes. The arkening shown in Figure 6 was less marked n glucolysed plasma with added glucose nan in normal plasma. This shows again nat other substances than glucose contribute o the increase in optical density. The exact ature of these substances is unknown.

From what has been said so far it is pparent that the time allowed for the reac-

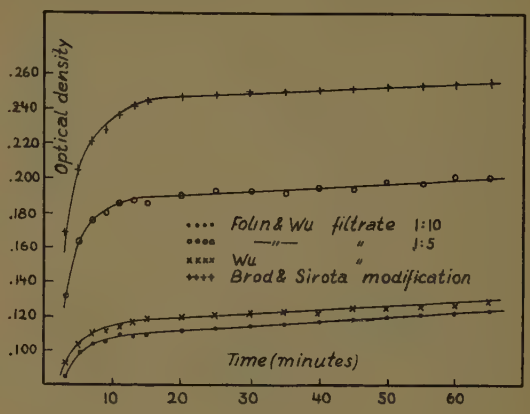

Fig. 6.

tion as well as the temperature at the reading, are factors affecting the absolute values of endogenous "creatinine". From Figures 3, 5 and 6 it will be seen that the concentrations of the deproteinizing agents influence the absolute values. The Wu filtrate gave a higher optical density than the Folin & Wu filtrate (1:10). The concentrations of endogenous "creatinine" in the Folin & Wu filtrate (1:5) and the Brod & Sirota filtrate are not directly comparable with the others because of the different degree of solution. Table II, however, shows the absolute concentrations of endogenous "creatinine" in 20 normal plasmas by using the four different concentrations of sodium tungstate and sulfuric acid mentioned above. It will be seen from the table that the Wu method gave higher values than the other three. The average value by the Wu method was 1.01 mg/100 ml, with a range of 0.77 to 1.18 mg/100 ml. In the Folin & Wu filtrates (1:10) the values were about 6 per cent lower (average 0.95 mg/100 ml, range 0.76 to 1.09). The lowest values were obtained in Folin & Wu filtrates (1:5) (average 0.88

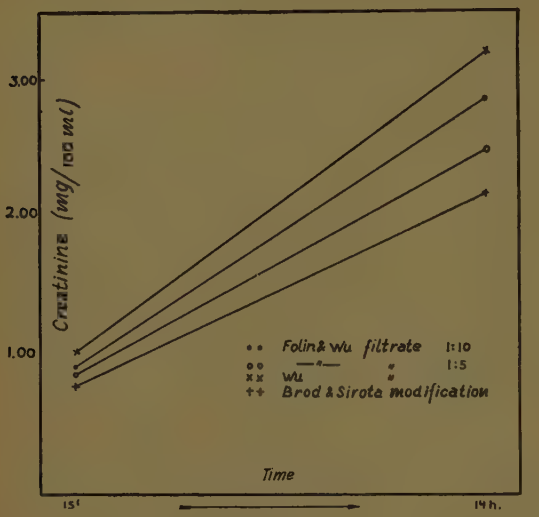

Fig. 7.

mg/100 ml, range 0.74 to 1.05). With the Brod & Sirota modification the average concentration was 0.91 mg/100 ml, with a range of 0.77 to 1.08 mg/100 ml, i. e. a value slightly higher than that obtained in Folin & Wu filtrates (1 : 5).

The discrepancies found may be due to various circumstances. Endogenous "creatinine" incorporates true creatinine and the so-called "pseudocreatinine". It is possible that differences in concentration of sodium tungstate and sulfuric acid may give different yields of true creatinine and "pseudocreatinine". It is thus possible that the yield of true creatinine is the same by all four precipitation methods, while that of "pseudocreatinine" varies, or the opposite may be the case, viz. that the yield of "pseudocreatinine" is constant, while that of true creatinine varies. Finally, a combination of these possibilities may be considered.

For the purpose of elucidating this problem, known amounts of creatinine were

Table III. *Recovery (in per cent) of creatinine added to plasma (average of experiments).*

Wu precipitation	Folin & Wu (1 : 10)	Folin & Wu (1 : 5)	Brod & Sirota modification
93.6 (84.2— 102.9)	84.7 (78.5— 90.8)	78.1 (67.7— 84.0	100.4 (93.8— 107.9

added to plasma by exchanging some of t water used for the deproteinization wi aqueous solutions of creatinine (as describ by Brod & Sirota). The results of the experiments are shown in Table III.

The Wu method gave 93.6 per cent covery of added creatinine ,with a range 84.2 to 102.9 per cent. The Folin & W method (1 : 10) gave a somewhat poorer covery, viz. 84.7 per cent (range 78.5 90.8 per cent), while the Folin & Wu meth (1 : 5) gave the poorest recovery (78.1 cent, range 67.7 to 84.0 per cent). The b recovery was obtained by the Brod & Sir modification. By this method the recove was 100.4 per cent, with a range of 93.8 107.9 per cent. This result confirms findings of Brod & Sirota, who recover on the average 100.2 per cent of added cr tinine. This good agreement was obtain in spite of different methods (here satura picric acid, Brod & Sirota the Bonsnes Taussky modification). According to Sir & Kroop (1951) the Bonsnes & Tauss modification avoids the acid reaction obtain by the Brod & Sirota modification. This a

Table IV. *Recovery (in per cent) of creatinine added to plasma filtrates.*

	Wu precipitation	Folin & Wu (1:10)	Folin & Wu (1:5)	Brod & Sirota modification
	101.3	96.4	97.3	98.1
	99.6	99.3	99.7	98.6
	100.0	103.4	100.3	100.6
	100.0	99.6	99.1	97.2
	98.5	101.1	99.0	96.5
Average	99.9	100.0	99.1	98.2

eaction is, however, of no consequence for ne following reasons: firstly, the Brod & irota modification gave a 100 per cent reovery of added creatinine, when using the aturated picric acid — 10 per cent sodium ydroxide method (as shown in Table III); econdly, it will be seen from Table IV that ne Jaffé reaction gave a reliable result in ll plasma filtrates, as a 100 per cent reovery was obtained when creatinine was dded to the *filtrates*.

A comparison between the four precipitaon methods is shown in Table V:

A further demonstration of the facts preented in Table V will be seen from Figure 7, ne results of which were obtained by reading ne color twice, i. e. at 15 minutes and 14 ours. The increase in optical density was ost marked in the Wu method, less marked the Folin & Wu method (1:10), still less arked in the Folin & Wu method (1:5), nd least in the Brod & Sirota method. This ccords closely to the conclusions drawn bove.

Table V. *Total "creatinine" values, average recovery (in per cent) and "pseudocreatinine" values by four different tungstic acid protein precipitation methods.*

Method	Total „creatinine" values	Average recovery of creatinine added to plasma	„Pseudocreatinine" values
Wu precipitation	High	93.6 %	High
Folin & Wu 1:10	Intermediate	84.7 »	Relatively high
» 1:5	Low	78.1 »	»
Brod & Sirota modification	Low	100 »	Low

DISCUSSION

The present study of the Jaffé reaction shows an increase in optical density dependent on temperature. The exact cause of this is not apparent, but changes of temperature must be considered a source of error in determinations of absolute values of endogenous "creatinine" in plasma and urine.

The results show that the color of aqueous solutions of creatinine and dilutions of normal urine can be read at any time between 15 and 65 minutes after addition of alkaline picrate. In plasma filtrates the color will darken for at least 65 minutes (according to Hayman, Johnston & Bender the optical density will increase indefinitely, as also indicated by the results shown in Figure 7). It seems reasonable that the color development during the first 10 minutes to a major extent depends on true creatinine, while the increase in optical density after this time depends on "pseudocreatinine". This accords with the view advocated by Hunter & Campbell. However, the results disagree

with those of Hunter & Campbell who stated that glucose was the only non-creatinine chromogen.

On the other hand, the fact that a greater part of the non-creatinine chromogen is glucose, explains why urine normally contains little or no" pseudocreatinine", as glucose is normally reabsorbed by the tubuli. Glucosuria will affect the Jaffé reaction in urine, and it is advisable to examine urine specimens for glucose, before making an endogenous "creatinine" clearance determination.

(It is interesting to note that a patient with diabetic renal disease recently observed in our department, on two examinations showed an endogenous "creatinine" clearance amounting to 170 ml/minute in spite of raised plasma "creatinine" and blood urea. I believe the reason for this is the glucosuria.)

The effect of time on color development in plasma filtrates will have an influence upon the endogenous "creatinine" clearance figures, as there normally is no corresponding change in urine. Measurement of the color after a short interval will give higher clearance values than a later reading.

It was demonstrated that protein precipitation by different concentrations of tungstate and sulfuric acid gave different values for endogenous "creatinine". The experiments indicate that the method of Brod & Sirota is to be preferred. This method gives a 100 per cent recovery of added creatinine and very low "pseudocreatinine" values. Brod & Sirota found the endogenous "creatinine" clearance determined by their method equal to inulin clearance. This could only be correct if their method measured just the true creatinine. This is hardly so. On the other hand their method must give an endogenous "creatinine" clearance not much h low inulin clearance on account of the lo "pseudocreatinine" value.

Next to the Brod & Sirota filtrate the W modification is to be preferred, and especial at greatly raised plasma "creatinine" conce trations, as the color otherwise will be dark for the colorimetration.

This study serves to explain some of t difficulties involved in the determinations endogenous "creatinine". It also explai why the Brod & Sirota method gives a lo endogenous "creatinine" plasma value. Br & Kotátko suggested that the low avera endogenous "creatinine" values in Brod Sirota's material were due to a low "pseud creatinine" value in U. S. A. This is o viously not so. The reason is found in t protein precipitation method.

The concentration of endogenous "cre tinine" depends on a number of factors su as the deproteinizing agents, the temperatu at the moment of color measurement and t reading time.

SUMMARY AND CONCLUSIONS

The present study draws attention to c tain factors involved in the determination endogenous "creatinine" in plasma and uri

1) The temperatures of the solutions m be kept constant during the colorimetrati and corresponding to that used for worki out the calibration curve.

2) Aqueous solutions of creatinine a dilutions of normal urine can be colo metrated at any time between 10 and minutes after the addition of alkaline picra Plasma "creatinine" values should be r at a constant time interval after the addit

the reagents as a continuous increase in tical density will occur. A considerable rt of this increase is due to glucose. The nsnes & Taussky modification gives the ne increase in optical density as the ori- nal Jaffé reaction.

3) The method of deproteinization is very portant. The Brod & Sirota modification the Folin & Wu method is the most liable and gives low "pseudocreatinine" lues. The folin & Wu method (1 : 5) gives ghtly lower values of endogenous "crea- ine", while the Folin & Wu method : 10) gives somewhat higher values than e Brod & Sirota modification. The highest lues are obtained by the Wu method, which so gives the highest "pseudocreatinine" lues.

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THE RENAL RESPONSE TO HEXAMETHONIUM

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INTRODUCTION

I 1949 Aas & Blegen showed that intravenous administration of tetraethylammonium bromide (TEAB) in doses of 4—6 mg per kg of body weight had distinct effects on the kidney. TEAB was injected very slowly in order to avoid the influence of a change in blood pressure. In more than half the number of cases a pronounced decrease in diuresis was produced. Another effect of TEAB was a decrease in renal plasma flow, i. e. a vasoconstriction. These two responses were at least partly independent of each other.

A vasoconstrictory effect of TEAB was the inverse of what is known to be the general effect of this drug; blocking of autonomic ganglia by TEAB leads to general vasodilatation. The inverse effect on the renal circulation is of considerable interest. It brings to mind the fact that physiological and pathophysiological studies have shown a frequent need for inverse relation between renal and general circulatory responses. An increased extrarenal blood flow is sometimes, at least partly, accomplished by a shut down of the renal circulation. This may happen in valvular heart failure and in experimentally induced hypoxemia (Blegen & Aas, 1950).

With regard to the inhibitory effect of TEAB on diuresis, the explanation remains unkonwn.

Another drug which is known to bl autonomic ganglia is hexamethonium. T drug is chemically related to TEAB, and also been used in the treatment of arte hypertension because of its ability to prod vasodilatation and to cause a fall in bl pressure. Hexamethonium acts a little m slowly than TEAB (Bechgaard, Iversen Nielsen, 1951) and decidedly less regula This is especially observed in patients fering from arterial hypertension. For reason it is to be expected that the r response of hexamethonium is less d spicuous than the effect of TEAB.

As very little is known about the r effect of hexamethonium, only one r tively incomplete statement being publis (Blainey, 1952), it is the object of present paper to follow this up.

We have examined the renal respons intravenous or subcutaneous administra of hexamethonium in eight individuals, t men and five women, age between 23 an years. One had only one functioning kid the other being aplastic, but blood pres was normal. Three, one man and women, suffered from arterial hypertens This is in contrast to the study of the e of TEAB, where only normal individ were selected. Now we wanted to inc some patients with arterial hyperten

Table I. *Effect of hexamethonium on diuresis and renal function in 5 normotensives.*

	Periods	Urinary flow (ml/min)	p-amino-hippurate clearance (ml/min)	Inulin clearance (ml/min	Filtration fraction (per cent)	Blood pressure	
1. K. L. ♂ 38 years	1	15.9	818	147	18.0	115/85	Control periods
	2	15.2	841	146	17.4	115/85	
	3	9.5					
	4	6.2					
	5	2.9	772	138	17.9	115/85	Vegolysen 25 mg subcutaneously just before end of period 2
	6	2.4	763	131	17.2		
2. M. F. ♀ 23 years	1	8.2	687	106	15.4	115/70	Control periods
	2	2.6	571	90	15.8		
	3	2.0					
	4	1.2	571	85	14.9	110/65	Vegolysen 25 mg subcutaneously at the beginning of period 2
	5	1.2	745	112	15.0	110/70	
3. M. G. ♀ 46 years	1	9.4	538	103	19.1	105/65	Control periods
	2	9.8	499	105	21.0		
	3	9.0	449	89	19.9	95/55	Vegolysen 10 mg intravenously at the beginning of period 3
	4	5.1					
	5	0.6	238	46	19.3	100/60	
4. T. G. ♀ 49 years	1	11.7	564	108	19.1	120/80	Control periods
	2	12.0	559	100	17.9		
	3	12.0	588	99	17.3	115/80	Vegolysen 6 mg intravenously at the beginning of period 3
	4	10.3	544	95	17.5	110/80	
5. H. J. ♀ 26 years	1	6.8	423	99	23.4	105/70	Control periods
	2	9.2	438	94	21.5		
	3	10.2	487	98	20.1	100/70	Vegolysen 10 mg intravenously just before beginning of period 3
	4	10.0					
	5	9.3	420	105	25.0	100/70	
	6	9.1					

though we wanted to administer the hexa-ethonium in such a way that the blood ressure was on the whole unaffected.

METHODS

Glomerular filtration rate was determined as ılin clearance, renal plasma flow as p-aminohip-rate clearance at low plasma concentrations —3 mg/100 ml). A sufficiently large diuresis ıs promoted by drinking water. The experimental ocedure as to priming and sustaining infusion of inulin and hippurate, bladder emptying etc. was the same as described by Homer Smith et al. (1938, 1945). Inulin was determined by the diphenylamine method described by Corcoran & Page (1939) and Alving, Rubin & Miller (1939), p-aminohippurate by the method devised by Homer Smith et al. (1945) with the exception that the plasma proteins were precipitated by trichloracetic acid. Hexamethonium[1] was injected either subcutaneously or

[1] Hexamethonium (Vegolysen) was kindly supplied by May & Baker Ltd., Dagenham, England through Mr. Olav Naper, Oslo.

for the intravenous route through the rubber tube for the intravenous drip, about 15 cm from the cannula.

RESULTS

The results are listed in the two tables. It will be seen that a change in glomerular filtration rate and renal plasma flow due to an effect of hexamethonium can be detected in only three instances. There is no difference in this respect between normals and hypertensives. But in every instance where hexamethonium can be seen to have had an influence upon filtration rate and blood flow, it is that of depression. In no instance signs of renal vasodilatation can be detected.

In at least half the number of cases a distinct depression of the urinary volume will be observed.

One will also see that the effect of hexamethonium on the blood pressure is of a magnitude insufficient to account for the changes in renal function: In normals there was no change in blood pressure at all; in the hypertensive there was a slight fall in the systolic pressure only, while the diastolic remained practically unaltered. In no instance were sensations like headache, fainting, paræsthesias or visual disturbances experienced. These sensations are commonly met after administration of TEAB. There were no objective signs except the insignificant blood pressure fall in the hypertensives. Protein was not detected in the urine.

DISCUSSION

The tables clearly show that the renal response to hexamethonium is decidedly slower and less conspicuous than the response to TEAB. As mentioned above this is only to be expected. One must howe also remember that although the substan are chemically related, they are neverthel so different that a direct comparison regard to pharmacological effect is diffic in many respects, for instance in regard dosage. Nevertheless, the results seem allow two conclusions. Firstly: hexameth nium did not produce dilatation of the re vessels detectable by the p-aminohippur method. In some instances a vasoconstricti occurred, just as is the case with TEA Thus it is confirmed that blocking of au nomic ganglia leads to an inverse effect the renal circulation of what is known occur generally. It is pointed out in the troduction that this fact may have a wi significance, and be connected with the re tionship between renal and extrarenal culatory adjustments. Because of the re tively large renal blood flow, a shutting do as in cardiac failure or experimentally duced hypoxemia may play a beneficial for the general circulation, and perhaps versa. The contrariety between general renal vascular response to TEAB and he methonium may thus be of physiological pathophysiological importance. It can be stated that it is no use to try to obt renal vasodilatation in cases of renal ische by the administration of ganglion block agents.

Secondly: Hexamethonium and TE exercise the same depressing effect on urinary volume. On first thought this ef might be explained as due to the vasoc striction and the concomitant depression the glomerular filtrate. And in fact this been done by Blainey (1952) who also fo a decrease in urinary flow after hexamet

Table II. *Effect of hexamethonium on diuresis and renal function in 3 hypertensives.*

	Periods	Urinary flow (ml/min)	p-amino-hippurate clearance ml/min	Inulin clearance (ml/min)	Filtration fraction (per cent)	Blood pressure	
6. P. Ø. ♂ 49 years	1	10.0	538	116	21.5	205/130	Control periods
	2	8.5	521	108	20.7	210/130	
	3	11.0					
	4	6.5	386	85	22.0	200/135	Vegolysen 10 mg intravenously at the beginning of period 3
7. A. E. ♀ 52 years	1	7.4	292	74	25.3	200/120	Control periods
	2	11.9	297	74	24.9	200/115	
	3	9.7	300	71	23.7	180/110	Vegolysen 10 mg intravenously at the end of period 2
	4	11.0					
	5	7.7	266	62	23.3	160/100	
	6	2.7					
8. E. J. ♀ 36 years	1	10.0	120	25	20.8	230/130	Control periods
	2	11.1	146	28	19.2		
	3	9.2	123	26	21.1	280/140	Vegolysen 3 mg intravenously at the beginning of period 3
	4	6.6					
	5	5.6	113	24	21.2	230/130	
	6	5.3					

ium. But it was shown in Aas & Blegen's paper on TEAB that there is no direct relationship between the two types of response. And an analysis of the tables in the present paper shows an abrupt fall in diuresis several times without a corresponding fall in blood flow or glomerular filtration rate. This is seen in cases 2 and 7. Such observations count much more than examples of simultaneous variations as these may be purely accidental. We therefore reach the same conclusion as did Aas & Blegen: The depression of diuresis is at least partly independent of changes in blood flow and glomerular filtration rate. Now one must be aware that in clearance determinations of long duration a decrease in diuresis usually occurs. But on examining our results we feel that the decrease in diuresis after hexamethonium (and TEAB) comes earlier and often more abruptly than we have been accustomed to see during our clearance studies. We are therefore fairly convinced that the depression of the diuresis, at least in some instances, is caused by the drug itself.

With regard to the explanation of this phenomenon, we have come no farther than in 1949 when the study on TEAB was published. We can only raise the question: why do ganglion blocking agents like TEAB and hexamethonium depress diuresis?

CONCLUSION AND SUMMARY

The renal response to hexamethonium (Vegolysen, May & Baker) was examined in five normotensives and three hypertensives. In no instance was a renal vasodilatation observed, but in two instances there was

found a distinct reduction in glomerular filtration rate and renal plasma flow, and in two instances an abrupt fall in diuresis. These two types of response were not related to each other, and it is concluded that the diuretic depression exercised by hexamethonium (as by tetraethylammonium bromide) is mainly independent of changes in renal plasma flow and glomerular filtration rate. Renal vasodilatation can not be obtained by the administration of ganglion blocking agents of the type tetraethylamm nium bromide or hexamethonium. On th contrary: a vasoconstriction may take plac

The significance of the inverse relationshi between renal and general circulatory r sponse is briefly discussed.

The depression of urinary volumes aft administration of hexamethonium (and tetr ethylammonium bromide) remains u explained.

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BLOOD UREA AND PLASMA ENDOGENOUS »CREATININE« IN RENAL INSUFFICIENCY

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The great epoch of renal functional studies which was inaugurated about 25 years ago is coming to an end. Along many lines the work continues and will advance our knowledge. From the physiological point of view it can be said that the outlook is bright. But there can be no doubt that the expectations in regard to how clinical medicine should benefit from all this new physiological knowledge have been largely disappointed. The methods for measuring renal function may be useful in clinical medicine, but it is a hard fact that only a very limited number of clinicians in any country are so well acquainted with all the pitfalls and all the limitations that are involved in the practical use of these methods, and this greatly reduces their clinical value.

Our department has been interested in working out a clearance method which is so simple with regard to technical procedure that it can be used by any clinical laboratory and which is at the same time so safe and so easy to interpret that it can be useful to any trained internist. We have found the endogenous "creatinine" clearance able to fill these obligations. Further information will be found in an earlier publication (Blegen, Haugen & Aas, 1949).

On account of the many difficulties connected with performing a complete urea or endogenous "creatinine" clearance (first of all complete bladder emptying which is a conditio sine qua non) many clinicians will often order just a plain blood urea determination, or, as Addis recommended, a plasma "creatinine" determination. And it is certain that such a determination may give information of some use.

It is our experience, however, that a blood urea determination very often will mislead and that a determination of plasma "creatinine" is a safer procedure. It is the object of the present paper to show this.

MATERIAL AND METHODS

The material comprises 12 patients, all suffering from chronic glomerulonephritis or pyelonephritis. Details regarding age, diagnoses etc. will be found in the table.

Blood specimens for urea and endogenous "creatinine" were obtained in the morning before breakfast; heparin was used as anticoagulant.

Urea was determined by Van Slyke's manometric method, endogenous "creatinine" was determined by the Jaffé reaction (saturated picric acid and 10 per cent NaOH were used). For the determination of endogenous "creatinine" plasma protein was precipitated by the Brod & Sirota (1948) modification of the Folin & Wu method or, in case of high plasma endogenous "creatinine", above 2 mg/100 ml, by the Wu (1922) modification of the same method. For the colorimetration a Coleman Junior spectrophotometer was used. Readings at a wavelength of 500 mμ, after exactly 15 minutes, at 23° C (calibration curve constructed on readings at the same temperature).

Table I.

Subject, age and diagnosis	Date	Blood urea (mg/100 ml)	Urea clearance (ml/min)	Plasma „creatinine" (mg/100 ml)	Endogenous „creat." clearance (ml/min)	Diet etc.
A. H. ♂, 42 years						
Chronic glomerulonephritis	14/9 1951	32		2.0	78	
O. N. ♀. 51 years						
Chronic glomerulonephritis	22/8 1951	31	30	1.55	58	Low protein diet
E. W. ♂, 70 years						
Chronic glomerulonephritis	18/4 1951	30	17	1.76	35	Low protein diet
J. J. ♂, 67 years						
Chronic glomerulonephritis	20/2 1951	20	31	1.46	79	Full diet
A. H. ♀, 21 years						
Chronic glomerulonephritis	9/2 1951	23		1.43	45	Low caloric intake
E. P. ♂, 55 years						
Chronic glomerulonephritis	25/1 1951	36	45	1.76	85	Low caloric intake
	2/2 1951	26	31	1.44	76	
M. K. ♀, 60 years						
Chronic pyelonephritis	22/1 1952	37		1.52	50	Full diet
	25/1 1952	42				
	5/2 1952	30		1.38		
E. K. ♀, 55 years						
Chronic pyelonephritis	13/9 1951	291		14	3.6	Low protein diet
	2/10 1951	179				
	10/10 1951	165		5.5	5.0	Ample supply of fluid
	18/10 1951	224				
	30/10 1951	170				
	8/11 1951	55		3.1	10.0	
	19/11 1951	57				
	30/11 1951	33				
	30/11 1951	35		2.8		
	26/11 1951			3.0	16.0	
E. J. ♀, 36 years						
Chronic glomerulonephritis	20/3 1952	80		3.0	28	19/3 Full diet
	26/3 1952	60				22/3 Low protein diet
	29/3 1952	35				
	1/4 1952	35		3.04		
E. A ♂, 60 years						
Chronic glomerulonephritis	18/3 1952	33		1.68		Low caloric intake
	20/3 1952	35		1.76		
R. B. ♂, 24 years						
Chronic glomerulonephritis	28/11 1951	39		1.83	44	Low protein diet
	5/12 1951	36				
	7/12 1951	46	14			
	28/12 1951	41	32	1.95	57	
W. R. ♂, 55 years						
Chronic glomerulonephritis	13/7 1951	53				Low protein intake
	21/7 1951	73				
	28/7 1951	71				
	4/8 1951	29				
	9/8 1951	30	29	1.8	44	

RESULTS

The results are listed in the table. A few etails will be pointed out. The patient E. K. ho suffered from a chronic pyelonephritis, as admitted in a bad condition. Her blood rea was 291 mg/100 ml and endogenous lasma "creatinine" 14 mg/100 ml. She was ut on a low protein diet and given an ample upply of fluid. After 5 weeks her blood rea had fallen from 291 to 33 mg/100 ml, figure which is regarded as "normal". lasma "creatinine" had fallen from 14 to bout 3 mg/100 ml, which is more than twice ie upper normal limit. She has been fol- owed for more than 6 months since, and creatinine" has never been found below mg/100 ml.

Much the same applies to patient E. J. ho at admittance had a blood urea value f 80 mg/100 ml and a plasma "creatinine" f 3 mg/100 ml. During her stay in hospital ie blood urea decreased to 35 mg/100 ml, hile plasma "creatinine" remained at the riginal high level.

It will be seen from the table that all lasma "creatinine" values are above the pper normal limit while the blood urea alues are normal or come down to normal gures on a low protein diet.

It will also be seen that the urea clearances re reduced in spite of normal blood urea, nd that there is better conformity be- ween endogenous "creatinine" clearance and lasma "creatinine" than between urea clear- nce and blood urea.

DISCUSSION

The upper normal limit for plasma "crea- nine" in our hands lies at 1.3 mg/100 ml. uring 5 years we have performed several ousands of endogenous plasma "creatinine" determinations, and we have never observed values above 1.3 mg/100 ml except when clearly explained by renal disease, cardiac failure, dehydration etc.

Regarding the "creatinine" determination there are several important factors to consider, viz. plasma protein precipitation, reagents, time of colorimetration, temperature of solutions at colorimetration (Haugen, 1953). Regarding "normal" blood urea we have considered 35 mg/100 ml as the upper limit, although even higher values may be considered "normal" on high protein consumption (Addis, Barrett, Poo & Yuen, 1947). All possible regard to accuracy at the determinations must be taken also here. Our urea determinations have been performed by trained laboratory personel, double determinations and with intervals checked by the Conway method.

Earlier authors have stated that in renal insufficiency "pseudocreatinine" may account for a greater percentage of total plasma creatinine than normally. It is however our experience that "pseudocreatinine" is significantly raised only in uremia, and none of the patients listed here except one, were in that state. At the plasma "creatinine" values seen in our table "pseudocreatinine" is too low to affect our conclusions.

The reason for the discrepancy between the course of blood urea and plasma "creatinine" is of course the well known fact that the blood urea level highly depends on the protein intake and that the plasma "creatinine" level does not. In regard to blood urea and protein intake so much has been written that we here will refer only to the rather recent report by Addis et al. and the detailed discussion of the problem by Peters & Van Slyke (1946). In regard to plasma

"creatinine", Barrett & Addis (1947) found no significant change in serum "creatinine" concentration on widely varied protein consumption. In renal disease a raised plasma "creatinine" value always signifies renal insufficiency, and in chronic renal disease such a figure will rarely become normal.

On the other hand when a patient suffering from renal disease is put on a low protein intake blood urea may often fall to normal values in spite of severe reduction in renal function. A normal blood urea value does not in itself signify that the kidneys function normally.

As a screening test a determination of plasma "creatinine" is therefore a better method than the blood urea determination. The same applies to the use of the two methods in following the course of the disease. It is of course important to remember the limitations of the value of using the blood level of any substance in judging renal excretory capacity. This subject has however been so extensively covered by earlier authors that it will not be discussed here.

Although the ideas presented in this paper are already known, we consider it of practical value for the clinician to get a demonstration of the fact which has been underrated or forgotten: in renal insufficiency the plasma endogenous "creatinine" level is a better measure of renal failure than the blood urea level.

It has interested the authors, who for many years have been devoted to clearance studies, that in renal disease a plain determination of plasma "creatinine" often w tell the clinician all he needs to know abo the degree of renal failure.

CONCLUSION

In renal insufficiency the finding of normal or even low blood urea level m lead the clinician to assume that there is reduction of renal function. This is not As a screening test and for following t course of renal disease determination plasma "creatinine" is a better procedu Plasma "creatinine" is largely independe of the protein consumption and therefo more dependent on kidney function than blood urea.

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THE TRUE ENDOGENOUS CREATININE CLEARANCE

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The use of inulin clearance as a measure glomerular filtration rate has certain disvantages: Inulin must be administered inavenously, it is expensive, and at least in candinavian countries ,it is very difficult to tain ampules of inulin for intravenous use. he reason is that making a pyrogenfree lution of inulin is so expensive that none the Scandinavian pharmaceutical firms ems it profitable.

But the most serious objection is the techcal difficulties. On one side there is the fficulty of keeping the blood level of inulin nstant for a prolonged time through the rmanent infusion technique. It can be done, t the procedure will always represent an stacle. Still more serious is the fact that e chemical analysis of inulin is difficult. omer Smith (1951) himself states that no rfect method for the determination of ulin exists.

Endogenous "creatinine" (total chromogen) clearance can be used as a measure of omerular filtration rate, and for practical e in clinical medicine this method gives fficiently exact results (Blegen, Haugen Aas 1949). But endogenous "creatinine" arance can not directly replace inulin clearce. The reason is that there are in plasma so other substances than creatinine which ve identical color in the Jaffé reaction. These extra substances are called "pseudocreatinine", and because with the usual procedure they are added to the real plasma creatinine value, but mostly not excreted in the urine, endogenous "creatinine" clearance will fall a little below inulin clearance.

If "pseudocreatinine" in some way is eliminated from plasma, the Jaffé reaction will be given only by true creatinine, and endogenous creatinine clearance and inulin clearance should give identical values. The object of the present study is to examine this question by comparing inulin clearance and true endogenous creatinine clearance.

Many attempts have been made to eliminate "pseudocreatinine" during the creatinine analysis. Miller & Dubos (1937) made use of enzymatic splitting of true creatinine and determined the concentration of remaining "pseudocreatinine"; by subtracting this from the total value they got the true plasma creatinine. With this method Miller & Winkler (1938) found good agreement between endogenous creatinine clearance and inulin clearance. The enzymatic method for true creatinine determination is, however, as elaborate as the inulin method and demands creatinine splitting microbes.

Another method for separation of true creatinine and "pseudocreatinine" is to make use of creatinine adsorbing reagents. Such

ragents are kaolin and Lloyd's reagent. Brod & Kotátko (1949) found good agreement between true endogenous cratinine and inulin clearance, when they used Lloyd's reagent. But the spreading in their relatively small material was considerable.

Also Hare, Goldstein, Barnett, McNamara & Hare (1949) made use of Lloyd's reagent and found an average true creatinine/inulin clearance ratio equal to 1.03 (range 0.82 to 1.26). Some years ago Brod (1949) gave us the idea of using Lloyd's reagent. This product, which is a hydrated aluminum silicate, may vary considerably. Some brands may not adsorb creatinine at all. We finally found that the Lloyd's reagent manufactured by Hartman-Leddon Co. and sold by Arthur H .Thomas Co., Phila., Pa., U. S. A. gave us very good and constant results. There is no difficulty in importing this reagent too Scandinavian countries. It is inexpensive.

METHODS

In 18 individuals in altogether 43 clearance periods we have determined inulin and true endogenous creatinine clearances simultaneously. Of the 18 individuals 10 were normals. The other 8 suffered from various diseases, viz. arterial hypertension, hyperthyroidism, nephrolithiasis, and chronic nephritis of slight or moderate degree. The reason why more severe cases of chronic nephritis were excluded, will be explained below. Some of the determinations were done in connection with studies of the renal response to hexamethonium (Haugen & Blegen 1953).

The experimental conditions were the often described, including priming and sustaining infusion of inulin,[1] indwelling catheter in the bladder, emptying of the bladder by expression and injection of water and air. The clearance periods varied from 10 to 20 minutes. Thirty minutes at least was u for reaching equilibrium. Heparin was used an anticoagulant.

At first we made use of the method of Brod Kotátko (1949) for determination of true cr tinine. In this method the true creatinine is sorbed to Lloyd's reagent by shaking, and "pseu creatinine" is determined in the supernatant fl True creatinine is the difference between t "creatinine" (total chromogen) and "pseu creatinine". But we found this method unsa factory. The reason is that the "pseudocreatini value is very small and therefore difficult determine exactly. For that reason we modi a method described by Hare (1950). The pro dure was: Plasma protein was precipitated sodium tungstate and sulfuric acid according Wu (1922). To 10 ml of plasma filtrate or dilu urine in centrifuge tubes were added one ml saturated oxalic acid and about 200 mg of Lloy reagent. The tubes were stoppered and occasi ally inverted for 10 minutes. The tubes were t centrifuged for about 10 minutes and the cl supernatant, containing the "pseudocreatinine", c fully aspirated off and discarded. To the tu were then added 10 ml of picric acid — sodi hydroxide solution (5 parts of saturated picric a 1 part of 10 per cent sodium hydroxide solut 12 parts distilled water). The original stop were replaced, the tubes well shaken to susp the Lloyd's reagent and allowed to stand for minutes with occasional inversion. The tubes w then centrifuged for about 5 minutes. The perfe clear fluid was used for colorimetration. W treated the same way as the unknowns was t as the blank. We made use of a Coleman Ju spectrophotometer, wavelength 500 mμ. A spe calibration curve for this method was worked the colors were read at 23° C.

Endogenous "creatinine" (total chromogen) determined by the Jaffé reaction, using satur picric acid and 10 per cent sodium hydroxide s tion. The colors were read after exactly 15 m tes, and care was taken regarding constant temp ture of the solutions.

Inulin was determined by the diphenylar method as described by Corcoran & Page (1 and Alving, Rubin & Miller (1939).

[1] Inulin was kindly supplied by U. S. Standard Products Co., Woodworth, Wis., U. S. A.

Table I. *Endogenous "creatinine"* (total chromogen), true creatinine and "pseudocreatinine" in plasma and urine.

	Endogenous "creatinine" (total chromogen) mg/100 ml	True creatinine mg/100 ml	"Pseudo-creatinine" mg/100 ml
rmal plasma ..	0.92	0.87	0.05
» ..	0.97	0.86	0.11
» ..	1.18	1.04	0.14
» ..	1.18	1.05	1.13
» ..	1.03	0.82	0.21
» ..	1.18	0.80	0.38
» ..	1.13	1.02	0.11
» ..	1.02	0.88	0.14
oritic plasma ..	1.35	1.00	0.35
» ..	1.76	1.58	0.18
rmal urine	0.80	0.81	
» (diluted)	0.78	0.79	
» ..	0.79	0.78	
» ..	0.80	0.79	
» ..	0.79	0.81	
» ..	0.81	0.80	

RESULTS

Table I shows the endogenous "creatinine" tal chromogen), true creatinine and seudocreatinine" in a number of indi-uals. It is seen that "pseudocreatinine" in sma varies between 0.05 and 0.38 mg/100 In two patients with impaired renal func-n the values were 0.35 and 0.18 mg/100 Most of our determinations show no seudocreatinine" in urine.

The relation between true creatinine clear-ce and inulin clearance in all 43 clearance iods is seen in the figure which shows a y good agreement. The average ratio for periods is 1.01 ± 0.07 (range 0.87 to 7). The coefficient of regression is 0.97, coefficient of correlation 0.93.

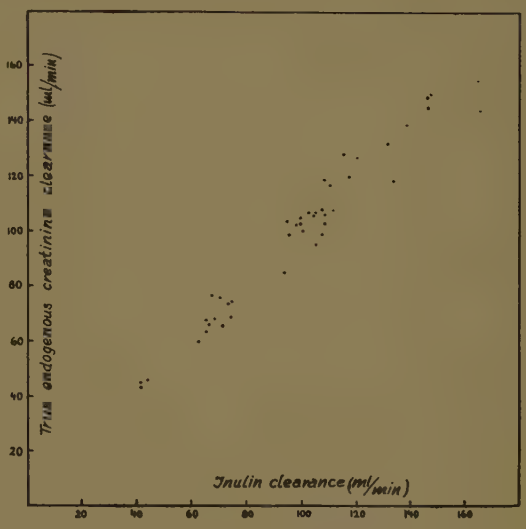

Fig. 1.

DISCUSSION

Our figures show a very good agreement between true endogenous creatinine clearance and inulin clearance with less spreading of the values than usually found in the literature. Some spreading is undoubtedly due to the inulin determinations, the disadvantages of which have been mentioned above. By using true endogenous creatinine clearance many of the difficulties connected with the inulin technique can be avoided. Not only is the determination of true creatinine clearance exact and easy to perform, but a great advantage is that the plasma level of true creatinine is very constant throughout the day. In shorter experiments only one plasma value is necessary.

Our results are a little better than those of Hare et al. We believe that the reason for this is our method for precipitation of plasma protein. Hare et al. made use of trichloracetic acid precipitation. In some experi-

ments we compared the two precipitation methods and found as a constant result that the trichloracetic acid precipitation gave a little lower true plasma creatinine value than the Wu precipitation. This will lead to higher clearance values just as Hare et al. found. Their ratio true creatinine clearance/ inulin clearance consequently was a little farther from 1 than in our material.

The object of this paper is above all to examine the relation of the clearances in normal individuals in order to find out whether true creatinine clearance under physiological conditions can replace inulin clearance. We are confident that this is the case.

When it comes to pathophysiology, things are not so easy. As shown in an earlier publication from our department (Blegen et al. 1949) and also by others (Brod & Kotátko 1949, Miller & Winkler 1938) endogenous "creatinine" (total chromogen) clearance in patients with seriously impaired renal function will tend to lie above inulin clearance. The ratio, which in the present study is 1.01, may be raised to 1.40 or even higher. In our earlier paper this was discussed and we concluded that the reason is that at high plasma "creatinine" levels s creatinine is excreted by the tubuli. Bu becomes more and more clear that in se renal disease all clearances are more or changed. In renal pathophysiology we longer have any "standard of referen There is no longer any possibility of show whether the excretion of a substance g a true measure of glomerular filtration or not. What the clinician needs is a met which he can learn to master, and here endogenous "creatinine" (total chromog clearance has distinct advantages.

CONCLUSIONS AND SUMMARY

A comparison between true endoge creatinine clearance determined by the of Lloyd's reagent and inulin clearance been made. In 43 clearance periods i normals and 8 patients with various dise but without seriously impaired kidney f tion the ratio true endogenous creati clearance/inulin clearance was 1.01 ± (It is concluded that under physiological ditions the true creatinine clearance eq inulin clearance and may replace it.

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PAPER PARTITION CHROMATOGRAPHY IN THE STUD OF KETO ACIDS

II. REDUCTION OF HYDRAZONES TO AMINO ACIDS

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In the studies on the paper chromatographic separation of the hydrazones of naturally occurring keto acids, it was found that besides α-ketoglutaric and pyruvic acid hydrazones also other hydrazones were present (Kulonen, Carpén & Ruokolainen, 1952). This paper will present experiments made in order to elucidate the unknown acid hydrazones.

The keto acids were isolated as 2,4-dinitrophenylhydrazones, separated with paper partition chromatography and the fractions eluted with ethanol. The hydrazone solutions were treated with aluminum amalgam in order to obtain corresponding amino acids by reduction (Groh & Fischer, 1911). The amino acids were investigated with paper chromatography.

EXPERIMENTAL

Isolation of the hydrazones. — Twenty liters of pooled human normal urine were evaporated under reduced pressure at a temperature below + 40° C to the volume of about 3 liters. The precipitate consisting of pigmented urates was removed by centrifugation. Sulphuric acid was added as a preservative in the amount of 0.5 ml per 100 ml. The concentrated urine was treated with 300 ml of 10 per cent sodium tungstate solution and with 300 ml of 10 per cent (by volume) sulphuric acid. The precipitate so formed was removed by filtration after 24 hours. Now 20 g of 2,4-dinitrophenylhydrazine was added as a warm saturated solution in 2 N sulphuric acid. A standing for 24 hours the hydrazone was extra three times with peroxide-free ether and the extr (1.2 liter) were combined. The ether solution extracted twice with 10 per cent sodium carbo solution (total 1.0 liter) and the extract wa once with 300 ml of ether. The carbonate solu was acidified with sulphuric acid at room temp ture. Besides precipitated hydrazones some brown masses were also formed. They were moved, although they also probably conta significant hydrazones. The final hydrazone paration was obtained by repeated extractions ether. The combined extract (1.0 liter) was centrated under reduced pressure to the vo of 200 ml and then dried with exsiccated so sulphate. Finally the ether was completely orated in vacuum and about 0.2 g of dark ma resulted.

Separation of the hydrazones. — The prepar described above was dissolved in 20 ml of glycine buffer at pH 7.6. However, the buffer not able to maintain the hydrogen ion conce tion and sodium hydroxide was added to this

Experiments were made using tertiary alcohol/ethanol/water (50 : 10 : 40) mixture as vent. The hydrazone solution was pipetted line of spots on the upper margins of the s of Whatman No. 1 paper and the spots chromatographed in the descending system. spots were cut off and eluted with ethanol. volume of the eluates was then reduced to 1 by evaporation. These solutions were used i following experiments. The spots are liste Table I.

Chromatographic experiments were also ducted using glass tubes tightly packed with

.ble I. *Formation of Amino Acids from Keto Acid Hydrazones by Reduction.*

lydrazones of Keto Acids	Amino Acid Obtained by Reduction
o.10 α-Ketoglutaric acid	Glutamic acid (faintly)
o.20 Two separate faint spots (unknown)	None with certainty
o.35 Pyruvic acid	Alanine
o.40 Faint spot (unknown)	Glycine
o.50 Derivative of pyruvic acid hydrazone	Alanine and serine
o.60 Two faint spots (unknown)	Spots indefinite[1]
o.80 Phenylpyruvic acid	Spots indefinite[1]
o.90 Free hydrazine, neutral hydrazones, unknown substances	Serine

Possible are leucine, valine, tyrosine and phenylalanine.

ed Whatman No. 1 paper. The solvent was ced through the column by suction. The results re encouraging but not superior to the proure described above.

Reduction of the hydrazones to amino acids. — uminum amalgam was prepared in the following y. Very fine chips of aluminum were treated h 5 per cent solution of sodium hydroxide until intense formation of hydrogen began. The chips re quickly rinsed in water and then treated for min. with 0.5 per cent solution of mercuric oride. The treatment with sodium hydroxide and rcuric chloride was repeated. The amalgam was shed in turn with water, ethanol and ether and s stored under petroleum ether.

Five ml of the ethanol solutions of the hydrazone ctions were treated for 40 hours with aluminum algam at room temperature. Water was added portions to make the final volume about 20 ml. e formation of hydrogen was moderate. The uminous mass of aluminum hydroxide was shed twice with warm water, and the washings combined. Their volume was reduced by evaporation at + 60° C to 0.5 ml. This solution was used for chromatography.

Twodimensional chromatograms were run with ethanol and phenol/NH_3. Glutamic acid, serine, glycine, alanine, valine and histidine were used as references. Controls were made from the hydrazone solutions before the reduction but they were all negative.

Experiment with blood serum.— An experiment was conducted similarly with 2 liters of bovine blood serum. The whole sample of hydrazones was divided into two parts and the one part submitted to reduction.

RESULTS

The results regarding the urine sample are presented in the table. From the hydrazones, which were prepared from the blood sample, alanine and tyrosine were obtained.

COMMENTS

The reduction in this form is not a satisfactory procedure for the investigation of α-ketoglutaric acid or of other dicarboxylic keto acids. The formation of serine from the pyruvic acid derivative indicates the -C-O- bond in β-C-atom of pyruvic acid, such as in the products of aldole condensation. The liberation of serine from the last fraction might have been due to some pyruvic acid derivative, which had been condensed in a manner similar to pyruvic acid. The formation of glycine indicates the presence of glyoxylic acid hydrazone, which has hitherto been uncertain. The breakage of larger molecules to glyoxylic acid may also been possible but it was not observed. The probable presence of keto acids deriving from valine or leucine could not be confirmed but the finding was not conclusively negative.

SUMMARY

The formation of amino acids from keto acid hydrazones, prepared from urine and blood serum, was investigated by reduction with aluminum amalgam. There were obtained glutamic acid, serine, glycine and alanine from the urine sample, tyrosine and alanine from the blood serum sample. The pyruvic acid forms products of aldole condensation during the preparation.

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ON PHOSPHATES AND PHOSPHORIC ACID S DIETARY FACTORS IN THE CALCIUM BALANCE OF MAN

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It is well known that the skeletal calcium n be drawn upon in the acid-base regula- on of the organism. In the days of severe abetic ketosis extreme values for calcium the urine were frequently observed. Early xperiments with mineral acids also indicate at the skeletal calcium can be used for eutralisation of excess acid. Ammonium loride has been used for the study of xperimental acidosis, and a heavy load of mmonium chloride is followed by an in- ease in the urinary calcium already on the rst day.

The most instructive experiments in this irection are those reported by Dennig al. (1929), Farquharson et al. (1931) and lbright & Reifenstein (1948).

Phosphoric acid and phosphates as intro- uced by variations in the composition of iets can be thought to influence Ca-meta- olism in two ways. Phosphoric acid in xcess might carry calcium with it in the ine for neutralisation purposes. Phosphates hich readily form insoluble Ca salts above certain pH might reduce the absorption of lcium.

A number of earlier studies have given arying results. Farquharson et al. (1931), their well known studies of calcium meta- olism on low Ca diets, observed no effect of high doses of neutral or acid phosphates on the fecal or urinary calcium. In some experiments with varying protein level in the diet, an increase of the daily intake from 40 up to 100 g protein daily was followed by a considerable increase of urinary calcium.

McCance, Widdowson & Lehmann (1942) observed a slight increase in Ca absorption and an even slighter increase in urinary Ca following the transition of their subjects from a moderate to a very high protein diet.

Mellanby (1949) concludes that phosphates added to the diet depress the absorption of calcium. This contention is based on experiments in vitamin-D deficient puppies. However, the experimental evidence to support such a conclusion is weak. On the contrary, the material presented in his publication rather points to no effect of phosphate in these experiments. Baylor et al. (1950) gave 2015 mg P as acid phosphate by mouth daily for 24 days to a 57 year old osteoporotic female. No significant increase was observed in fecal calcium during phosphate administration, but marked reduction of urinary calcium occurred. The decrease in urinary calcium as compared with control periods was seen also when 534 mg Ca as gluconate was injected intravenously daily for 8 days during phosphate period. Significant decrease in urinary calcium was observed in children

following addition of sodium glycerophosphate to the low-calcium diet. Knapp (1947).

Recently Leichsenring et al. (1951) published experiments in which they observed that the addition of 600 mg P as dibasic phosphate reduced the calcium absorption by about 200 mg daily. They feel that variations in the phosphate content of the diet may be a factor of considerable importance in calcium metabolism.

These, as well as most other studies in man, were of short duration.

For some years, protracted Ca-studies have been carried on in this laboratory. The purpose has inter al. been to establish a better basis for the evaluation of the calcium requirement of man. The experiments will be reported in full later.

In the course of these investigations, which up to now have been conducted on 30 men in all (continuous observations for more than two years in some of them), many types of individual variations have been observed.

In the light of the recent publications on the effect of phosphates on the calcium balance, some studies were included in our investigations. Since the results of our studies indicate clearly that variations in the intake of phosphate or phosphoric acid, as can occur in various dietaries, have no influence on the calcium balance, a publication of these results seems warranted at this time.

EXPERIMENTAL

The experiments to be presented are the following.

1. Brief ingestion (one week) of 2.37 g orthophosphoric acid daily (750 g P) in four adult men (28, 29, 32 and 43 years of age).
2. Protracted ingestion (four weeks) of 3.16 g of orthophosphoric acid (1000 mg P) daily in two adult men (40 and 43 years of age).
3. Protracted ingestion (eight weeks) of 0.8 g orthophosphoric acid (250 mg P) daily in fo adult men (20, 28, 46 and 56 years of age
4. Protracted ingestion (twelve weeks) of neutral (Na -K) phosphate mixture (600 m P) daily in two adult men (20 and 43 years age).

All these experiments were carried out experimental persons who had been observ with regard to their calcium balance for mont and years prior to the experiments with ad tion of phosphoric acid and phosphate. T experiments were carried out under the mo careful control of the dietary intake.

5. In addition, the effect of a very high meat di (lean beef) was also studied in four oth adult men who were members of the technic staff (27, 28, 38 and 43 years of age) T daily food intake was measured roughly, and t variations were somewhat larger than in t phosphorus experiments.

It was calculated that the intake of prote was approximately 70—100 g. daily duri the control period. The protein intake w raised to 200—230 g daily during the sev days of experiment. The calculated increase the acidity of the diet was approximately 12 —1400 ml N/10 acid daily.

MATERIAL AND METHODS

Healthy male prisoners, 20 to 56 years of a have volunteered in the experiments reported. had been in the prison for some months bef joining the balance studies. They had been und careful medical supervision. Blood and urine a lyses and, in most cases, X-ray of the whole ske ton, established them as being normal, well-f individuals. No ill effects have been observ during the experimental periods, although t highest load (3.16 g daily) of orthophospho acid, administered thrice daily as reagent gra orthophosphoric acid (84 per cent) in 100 g apple jam, was felt as a strain.

The four members of the technical staff par cipating in the high meat diet-experiment (800 g lean beef daily for seven days) tolerated the li protein diet well.

DIET

The experimental diet for the prisoners has been repared by an expert dietician, and is weighed and rved under strict control. The diet is a good ixed diet. One hot meal is served daily, the menu ins on a 14 day schedule through all experi-ental and control periods. The bread is a standard e bread (80 per cent extraction), practically nytin-free. Skimmed milk is the main calcium pply. On the average the daily intake of protein as been 110 g. Foodstuffs rich in phytic acid or xalic acid have been carefully avoided.

Aliquots of the food as served have been analysed r calcium and phosphorus throughout the experi-ental periods. All excreta have been collected and nalysed through 14 day periods.

ANALYTICAL METHODS

Calcium and phosphorus determined on wet igested material (nitric acid-perchloric acid). Cal-um as oxalate by macro and micro permanganate tration, phosphorus photometrically after color development with molybdate and aminonaphtol-sulfonic acid.

pH in 24 hour urine, (preserved with thymol) by glass electrode.

Titrable acidity in urine by electrometric titration to pH 7.4.

Preformed urinary ammonia in 24 hour urine by Conway microdiffusion procedure.

Total base in urine by the method of A. Følling (Skand. Arch. f. Physiol. 1931, 21:27) slightly modified.

Potassium in urine by flame photometry.

CO_2-combining capacity of plasma by the Van Slyke manometric gas apparatus.

Unknowns included in all analytical procedures.

Table I. *Calcium in urine in four adult men following ingestion of 2.37 g orthophosphoric acid (750 mg P) daily for seven days. Values expressed as mg Ca/day.*

Exp. person	Control period before ingestion (average of 98 days)		H_3PO_4 (7 days)	Control period after ingestion (average of 42 days)	
	Mean	S. D.		Mean	S. D.
1	107	± 12	87	109	± 11
2	416	± 59	405	465	± 53
3	242	± 33	234	237	± 16
4	412	± 43	337	375	± 22

During H_3PO_4-period: Urinary pH dropped pproximately .2—.3 units. Total titrable acidity creased approximately by 150—200 ml n/10 acid. reformed ammonia increased by approximately)—100 mg daily. Exp. diet without phosphoric id gave a urinary pH of 6.0—6.4, and a titrable idity of 150—250 ml n/10 acid.

Table II. *Calcium in urine in two adult men following ingestion of 3.16 g orthophosphoric acid (1000 mg P) daily for four weeks. Values expressed as mg Ca/day.*

Exp. person	Control period before ingestion (average of 98 days)		H_3PO_4 (28 days)	Control period after ingestion (average of 84 days	
	Mean	S. D.		Mean	S. D.
2	270	± 5	256	299	± 19
9	212	± 52	167	211	± 26

During H_3PO_4-period: Urinary pH dropped approximately .6—.7 units. Total titrable acidity increased approximately by 200—300 ml n/10 acid. Preformed ammonia increased by approximately 100—300 mg daily.

RESULTS

The results of the experiments with 2.37 g of orthophosphoric acid are presented in Table I. As in the experiments of Farquharson et al. with inorganic phosphate, a slightly diminished output of urinary calcium was observed. The phosphoric acid is excreted in the urine with the aid of some reduction of the pH, an increase in the titrable acidity

Table III. *The calcium balances of two adult men following ingestion of 3.16 g orthophosphoric acid (1000 mg P) daily for four weeks.*

Exp. person	Days	Intake	Urine	Feces	Balances	H_3PO_4 added
		Ca mg/day				
2	98	464	270	153	40	0
	28	423	256	114	53	3.16 g
	84	458	299	102	58	0
9	98	457	212	296	−50	0
	28	431	167	271	− 7	3.16 g
	84	459	211	223	26	0

The P-intake was approximately 1400 mg/day in the control periods, 2400 mg/day in the H_3PO_4-period.

and a slight increase in preformed urinary ammonia. No significant changes in the CO_2-combining capacity of the plasma, indicating metabolic acidosis, were found, nor were significant variations observed in the urinary excretion of potassium and total fixed bases. It should be noted that in P-balance experiments conducted continuously over months in these men, the ingested 750 mg P were recovered in the urine in the course of the experimental week and in the following week. Increasing the daily dose of orthophosphoric acid to 3.16 g for four weeks gave identical changes in the urinary excretion. The pH-lowering effect was slightly more marked, and so was the increase in titrable acidity and ammonia production. The data on urinary excretion and the calcium balances are found in Tables II and III.

The moderate dose of 0.8 g orthophosphoric acid given daily over 8 weeks had no effect on the calcium balance. The evidence in favor of this conclusion appears from Table IV. It should be noted that the 250 mg

Table IV. *The calcium balances of fo adult men in the course of eight weeks which 0.80 g orthophosphoric acid (2 mg P) was added to the diet daily.*

Exp. person	Days	Intake	Urine	Feces	Balance	H_3P adde
		Ca mg/day				
5	56	612	223	410	−21	0
	56	575	162	392	20	.80
	84	575	154	398	23	0
6	56	991	342	557	93	0
	56	965	317	556	82	.80
	42	985	295	511	179	0
7	56	620	454	267	−101	0
	56	579	401	237	−59	.80
	84	594	406	239	−51	0
8	56	973	229	621	123	0
	56	927	219	639	69	.80
	84	933	211	655	67	0

P were recovered in the urine during t experimental period.

In the experiments with 750 and 250 mg as orthophosphoric acid, very detailed studi were carried out during control and expe mental periods. These included blood an lyses of calcium, inorganic phosphorus, alk line phosphatase, hemoglobin, NPN, CO combining capacity and total serum protei In the urine the daily excretion of organ acids was also followed as was the nitrog excretion, but as no significant changes we found in either of the two series of expe ments, the analytical program was reduc during the other series here reported (expe ments with 1000 mg P as orthophospho acid, the neutral phosphate mixture and t high meat diet experiments).

Table V presents the experiments wi 600 mg P as neutral phosphate added to t usual diet. In one person the fecal calci

Table V. *The calcium balances of two adult men following the addition of 600 mg P as neutral phosphate daily to the diet for twelve weeks.*

person	Days	Intake	Urine	Feces	Balances	Remarks
			Ca mg/day			
	98	465	263	150	52	Average of 98 days, no P added
	84	448	205	146	96	Average of 84 days, 600 mg P added
	112	455	290	113	52	Average of 112 days, no P added
	98	558	206	418	— 66	No P added
	84	543	173	478	—108	600 mg P added
	112	529	168	621	—260	No P added

The P-intake in the control periods was approxitely 1400 mg/day, and 2000 mg/day in the P-iod.

slightly increased, in the other person it slightly reduced. In both men, some reduction of the urinary calcium occurred during e phosphate period. The Ca-balances to be culated from the given figures indicate at the balance was not influenced.

Table VI gives the results of the experients with the high meat diet. Only one of e four experimental persons showed a deite increase in the output of calcium in the ine.

DISCUSSION

The results here presented must be discussd in relation to: 1. The pathways of calum in the body, and 2. the experiments by evious authors who have produced a dinct effect of acids or acid producing salts the calcium excretion in the urine.

The one recognized effect of acid or d salts on calcium in the intestine is to increase absorption. It is now practically universally recognized that the body regulates its content of calcium only by the way of excretion in the urine. Anions which react with calcium to produce insoluble calcium salts may depress the absorption of calcium. Typical examples are oxalic acid and phytic acid.

As mentioned in the introduction, the results obtained by previous workers with phophates have varied. The results here presented are in agreement with the early work of Farquharson et al., but at variance with

Table VI. *The urinary Ca as influenced by 800 g of lean beef daily in four adult men.*

Exp. person	A	B	C	D	
			Ca mg/day		
	444	121	258	344	pre-control period (6 days)
	352	162	245	363	
	405	127	248	365	
	430	126	282	346	
	372	106	282	363	
	435	136	260	309	
Average:	406	130	263	348	
	461	126	326	308	meat period (7 days)
	388	92	343	436	
	420	133	293	393	
	382	169	180	536	
	430	143	185	354	
	339	186	238	418	
	444	190	196	515	
Average:	409	148	252	423	
	436	93	661	291	post-control period (6 days)
	336	137	222	298	
	253	128	290	286	
	305	176	268	295	
	330	137	225	190	
	315	121	130	259	
Average:	329	132	299	270	

the recent results of Leichsenring et al. (1951). These authors claim that one week is an adequate period for adjustment to a Ca intake as low as 300 mg. Data from the literature (Bauer, Albright & Aub, 1929) furnish support for this claim. The present author has recalculated the data on the normal controls of Bauer et al. together with the data given in their compilation of the literature. This recalculation has been made in order to obtain the variations in daily fecal calcium from one 6-day period to another. Only consecutive 3-day periods have been used. The result of the recalculation shows that of the 15 individuals with two to five consecutive 6-day periods on calcium intakes from 80 to 315 mg daily, 12 showed a small difference between the lowest and the highest daily fecal calcium, viz. less than 70 mg. Three individuals, however, showed a range of 110, 165, and 278 mg respectively. The one with a range of 110 mg in daily fecal calcium will be found in the paper by Sherman, Gillett & Pope (1918). The remaining two with a range of 165 and 278 mg respectively, are normal controls of Bauer et al.

It thus seems well substantiated that on very low calcium intakes, the majority of normal individuals will adapt themselves in the course of about one week and show rather small variations in fecal calcium.

However, experiments of the present author with higher calcium intakes in adult men, followed over many months, have shown that a sudden drop in daily calcium intake from say 900 mg to 500 mg and, on the other hand, a sudden increase of the same order of magnitude, may give very different responses in the fecal calcium in different individuals. In some, adjustment takes place during a 14 day period, in others it may ta several weeks. As in our experiments t changes in calcium intake are brought abo by altering the daily ration of skimmed mi a corresponding drop in phosphorus inta will necessarily follow reduction of the c cium intake.

The saturation of the body with calciu or in other words the daily need for calciu no doubt plays a major role in determini the length of the adjustment-period.

There is however another observatio reappearing rather frequently in our mater which must be considered in all experime on calcium metabolism. In the course experiments where individuals have be followed on the same level of calcium a phosphorus intake for many months, per dical variations in fecal calcium with o small changes in urinary calcium have b observed. These "waves" in the fecal calci excretion seem in our experiments not to related to season or vitamin-D fluctuati in the diet, but must at the present ti remain unexplained.

As technical errors can be excluded in experiments these periodical variations o weeks and months in the fecal calcium, m be a reality and cannot be disregarded further studies on calcium metabolism. T practical consequence of these observati for all studies of calcium requirement, for the individual reaction to sudden chan in calcium intake, is that conclusions ba on brief (one or a few weeks) experime must be drawn with modesty and great c

In balance experiments where new v ables are suddenly introduced, say by alter the calcium or the phosphorus intake, both, definite conclusions are permissi

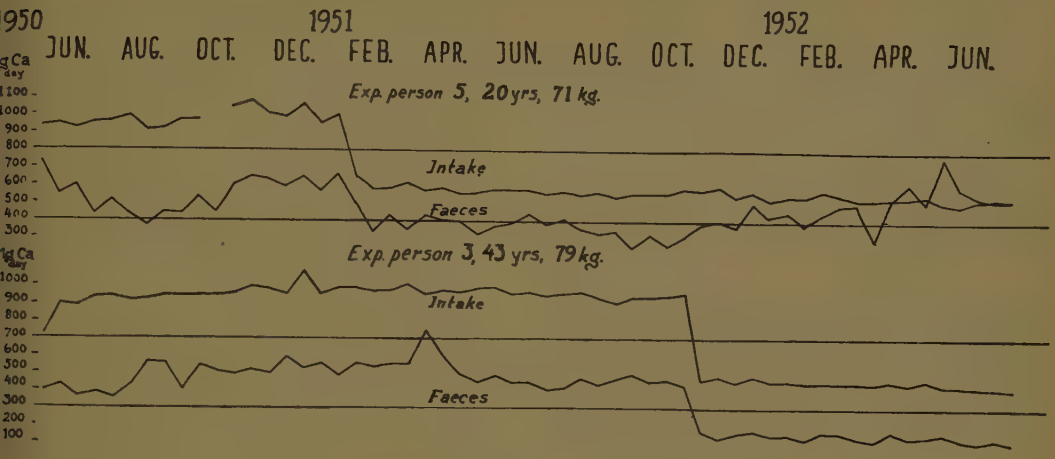

Fig. 1.

ly when the individuals in question have en observed for considerable time(months) a constant diet which will disclose indi-lual periodicity in the fecal calcium in ny normal adult individuals. It is then ssible to take into account these periodical anges in calcium absorption when con-usions are drawn. In brief experiments on eviously unobserved persons one cannot safeguarded against this capricious indi-luality in calcium absorption which might d investigators to erroneous conclusions. In order to illustrate this individual perio-city in fecal calcium excretion on constant akes of calcium and phosphorus, data for cium intake and fecal calcium are given Fig. 1. Two adult active men, living under e same conditions and on the same experi-ntal diet, have been followed for 24 and months respectively.

It will be noted that when the experiment s started with exp. person No. 3 (43 years age), significant variations in fecal calcium re observed on a liberal calcium intake 00 mg daily). After 16 months on the same diet, his milk intake was reduced. He adjusted himself during a 14 day period to the lower calcium intake and the fecal calcium showed only small variations during the next 8 months of experiment. This individual is therefore well suited for experiments with variations of phosphorus intake, the calcium intake remaining constant. The other type, which in our material is by no means rare, is represented by exp. person No. 5 (20 years of age). Large variations in fecal calcium were observed when he was put on a liberal calcium intake, the high calcium absorption during the first 5 months of observation indicating a real need for calcium. When his milk intake was reduced, he showed during the following 16 months marked periodical "waves" in the fecal calcium. These variations must make him less well suited for brief experiments, as it seems a matter of chance only whether short time balance studies would find him in a "high fecal calcium phase" or a phase with more efficient calcium absorption.

The observation of such periodical waves in fecal calcium excretion in certain individuals has been taken into account in our interpretations of the experiments reported. Considering next the experiments of Leichsenring et al., it must be questioned if it is warranted to draw the conclusions set forth in their paper. The authors take it for granted that when their experimental individuals adjust themselves over one week to an intake level of 300 mg Ca, they will necessarily do so when the intake is raised to 1500 mg. The opinion is ventured that unless they could demonstrate by protracted studies that their experimental individuals showed only small individual changes in fecal calcium on a high calcium intake (1500 mg), it is unsafe to conclude that the changes observed in fecal calcium are due to different levels of phosphate only. Spontaneous variations in calcium absorption not related to different intakes of phosphates over shorter periods, is another possible explanation which cannot be overlooked.

Turning next to the experiments with phosphoric acid, it follows from the above that the essential problem here is the urinary calcium.

It should be recorded that ammonium chloride, when it increases the calcium excretion in the urine, does so very rapidly. A marked increase is observed within 24 hours.

In the experiments of Farquharson et al., 100 m.e. ammonium chloride increased the urinary calcium by 200—300 per cent.

In the experiments with 24 m Mol. (2.37 g) orthophosphoric acid here presented, the result of one weeks experiment showed a decrease in urinary calcium in all four experimental persons. The decrea was slight in three cases, marked in one.

Experiments carried out later with 32 Mol. (3.16 g) of orthophosphoric acid ov four weeks gave a definite decrease in urina calcium in both experimental persons.

The results with the high meat diet we mostly negative. It should be noted that t calcium intake in these individuals was co paratively high, and also that the expe ments were conducted in healthy and phys ally active males, in contrast to the hos talized patients used by Farquharson et The striking increase in urinary calcium f lowing immobilization in bed is well kno

The protracted experiments with the 0. orthophosphoric acid in the four men quite conclusive as to the complete lack effect of this moderate load over 8 we time.

In the relevant series Leichsenring et studied the effect of phosphate on a diet c taining 1.5 g calcium and 0.8 and 1.4 g respectively. The extra P was ingested di-basic calcium and sodium phosphate. T compared the results of four week exp ments in two different groups of six pers each. The group on 0.8 g P absorbed on average 200 mg Ca more than the gr on 1.4 g P.

The general experience in our protrac studies on active adult men is that the f calcium can vary considerably both from 2-week period to the next and in lon waves lasting for several 2-week period

Recently a very thorough study of problem has been published by Bon (1952). He used very high doses of or phosphoric acid in rats over generations. results were entirely negative. No effec

-metabolism or the ash content of the bones as observed. Although care should be taken applying results from the ingestion of ven quantities of acids in rats to man, it is interest to note the striking similarity be- veen man and rat in their tolerance to phos- ioric acid.

Recent experiments on rats in this labora- ry (Nicolaysen & Mortenson-Egnund, 952) strongly support the conclusion that ide variations in the phosphate intake do ot affect the absorption of calcium. Two oups of twelve rats each were fed for four ccessive weeks on a diet with 0.5 per cent lcium. The phosphorus content was 0.34 d 0.57 per cent respectively, achieved by dition of neutral phosphate to the low-P et. The results were most consistent and owed the same absorption of calcium on fferent levels of P-intake. The experiments ere performed in young vitamin D -free ts which absorbed about 30 per cent of the lcium ingested.

SUMMARY

. 2.37 g H_3PO_4 (750 mg P) given daily for one week to four adult men engaged in active work, gave a slight decrease in urinary calcium in three cases, and a marked decrease in one case.

. 3.16 g H_3PO_4 (1000 mg P) given daily for four weeks to two other adults living under the same well-controlled conditions, reduced the urinary calcium slightly in the one, somewhat more markedly in the other case. The calcium balance remained unchanged by phosphoric acid administration in both cases. The calcium intake was about 450 mg daily.

3. 0.8 g H_3PO_4 (250 mg P) given daily for eight weeks to four other adult men under the same experimental conditions had no effect on the calcium balances. The calcium intake was about 950 mg daily in two cases, about 600 mg in the remaining two.
4. An increase of the P-level of the diet from 1.4 to 2.0 g of two active adult men (Ca-intake 0.5 to 0.6 g daily) had no effect on the calcium balances over a period of twelve weeks. The additional 600 mg P were given as a neutral Na-K phosphate mixture. A definite decrease in urinary calcium was observed during the P-period in both cases.
5. Increase of the P-content of the diet by adding orthophosphoric acid or neutral phosphate was followed by a reduction of urinary calcium in all experimental persons.
6. An increase (by lean beef) in the protein intake in the daily diet of four active adult men from approximately 70—100 g to 200—300 g for seven days was followed by a 20 per cent increase of the urinary calcium in one person, by somewhat slighter increase in a second person. In the other two no significant increase occurred.

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HE EFFECT OF DIHYDROERGOTAMINE AND HYDERGIN ON PULMONARY ARTERIAL PRESSURE IN MAN

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Recent studies (Cournand, 1947, 1950; amilton, 1951) on pulmonary circulation animously emphasize its relative refractori- ss towards vasoactive substances. Adrena- ie, nitroglycerine and other compounds hich deeply affect the peripheral vessels, do ot markedly influence the pulmonary arte- al pressure. Anoxia is the only agency hich is known to constrict the pulmonary scular tree (Westcott et al., 1951; Lilje- rand, 1948; Dirken & Heemstra, 1948; oyle et al., 1951) in man. The possibility a pharmacological vasoconstriction in the lmonary circuit is generally refuted.

Liljestrand 1948, however, pointed out at ergotamine raises pulmonary arterial essure in the cat. It was decided, therefore, investigate this effect in man. Because of untoward side-effects, two of its deriva- es dihydroergotamine (DHE) and hyder- n-were used.

METHODS AND MATERIAL

Investigations were carried out on 17 men and omen. Cardiac catheterization was performed in of the cases, in the usual manner. The details our technique were described in an earlier report Halmágyi et al., 1952). The tip of the catheter s placed in the right ventricle or in the pulmo- ry artery. Oxygen consumption was measured ctrically by using a Noyons-type of diaphero- ter (Kipp and Sons, Delft, Holland). Arterio- nous oxygen difference was determined photo- electrically (Issekutz Jr., et al., 1949). All pressure measurements were made with a Sanborn electro-manometer.

The determination of cardiac output was based on the Fick-principle and was calculated on one square meter body surface area (BSA/cardiac index).

The term "pulmonary arterial pressure" indicates in this study the systolic pressure of the right ventricle or that of the pulmonary artery.

Pulmonary and peripheral arterial systolic resistance was calculated by using the following equation (Riley et al., 1948):

$$R = \frac{P \times 1.332 \times 60}{CI}$$

where

R = systolic resistance in the pulmonary or systemic circuit, dyne/sec/cm^{-5}/m^2 BSA.

P = systolic pressure in the pulmonary or systemic arteries, mmHg.

CI = cardiac index, ml/min.

The statistical significance of the differences was calculated by the "table of "t"" (Fisher, 1934).

The dose of DHE was 1.0 mg, that of Hydergin 0.3—0.5 mg. Both substances were administered by a slow intravenous, intracardiac or intrapulmonary injection, by means of an electric pressure pump. These different ways of administration did not significantly modify their effect.

RESULTS

The effect of DHE is shown in Table I. Pulmonary artery pressure, systemic and pulmonary systolic resistance increased significantly, while mean systemic arterial pressure and cardiac output remained practically un-

Table I. *The effect of dihydroergotamine on cardiac output, systemic and pulmonary systolic arterial pressure and resistance in man.*

No.	Diagnosis	Peripheral art. syst. pressure mm. Hg.			Pulmonary art. syst. pressure mm. Hg.			Cardiac index lit/min.			Peripheral syst. resistance dyne/cm^{-5}/sec/m^2 BSA			Pulmonary art. syst. resistance dyne/cm^{-5}/sec/m^2 BSA		
		B	A	D	B	A	D	B	A	D	B	A	D	B	A	D
1	Aortic reg.	125	130	+ 5	22	33	+11	4.36	4.36	0.00	2290	2380	+ 90	403	607	+204
2	Mitral stenosis	115	110	− 5	21	30	+ 9	4.52	3.87	−0.65	2040	2275	+ 235	372	638	+266
3	Neurocirc. asth. ...	115	115	0	28	40	+12	5.07	5.00	−0.07	1815	1840	+ 25	422	639	+197
4	Neurocirc. asth. ...	120	120	0	23	28	+ 5	4.08	4.20	+0.12	2360	2290	− 70	452	533	+ 81
5	Cor pulm. chron ...	145	155	+10	28	34	+ 6	2.50	2.00	−0.50	4630	6200	+1470	896	1360	+464
6	Diab. mell.	130	145	+15	24	38	+14	3.81	3.71	−0.10	2740	3140	+ 400	505	820	+315
7	Sine morbo	105	115	+10	21	27	+ 6	6.03	6.15	+0.12	1395	1495	+ 100	279	352	+ 73
8	Mitral stenosis.....	135	135	0	27	39	+12	3.52	4.07	+0.55	3060	2650	− 410	614	770	+156
	Mean	124	128	+ 4	24	33	+ 9	4.24	4.18	−0.06	2560	2790	+ 230	495	715	+220
	Significance of differences	none			$t=8.03$ $P<0.01$			none			$t=2.656$ $0.02<P<0.05$			$t=7.562$ $P<0.01$		

Legend: B = before. A = after, administration of dihydroergotamine. D = difference between the two previous values.

Table II. *The effect of hydergine on cardiac output, systemic and pulmonary arterial systolic pressure and resistance in man.*

Nr.	Diagnosis	Peripheral art. syst. pressure mm. Hg.			Pulmonary art. syst. pressure mm. Hg.			Cardiac index lit/min.			Peripheral syst. resistance dyne/cm^{-5}/sec/m^2 BSA			Pulmonary art. syst. resistance dyne/cm^{-5}/sec/m^2 BSA		
		B	A	D	B	A	D	B	A	D	B	A	D	B	A	D
1	Diab. mell.	135	135	0	27	29	+2	3.60	4.05	+0.45	3000	2570	−430	600	573	− 27
2	Angina pectoris ...	120	100	−20	26	26	0	3.14	3.55	+0.41	3060	2250	−810	664	587	− 77
3	Mitral stenosis	140	130	−10	26	29	+3	1.97	1.95	−0.02	5680	5330	−350	1060	1190	+130
4	Cor pulm. chron. ..	110	110	0	26	30	+4	3.46	3.46	0.00	2550	2550	0	600	694	+ 94
5	d. Botalli persist. ..	115	105	−10	38	34	−4	4.23	5.07	+0.84	2180	1610	−570	718	537	−181
6	Aortic regurgit. ...	185	180	− 5	28	34	+6	3.05	2.77	−0.28	4850	5200	+350	735	984	+249
7	Mitral stenosis	130	120	−10	33	33	0	-	-	-	-	-	-	-	-	-
8	Mitral stenosis	90	85	− 5	59	47	−12	5.92	5.92	0.00	1220	1280	+ 60	799	632	−167
9	Mitral insuff.	115	132	+17	37	38	+1	3.16	3.16	−0.06	2910	3400	+490	937	980	+ 43
	Mean	127	122	− 5	33	33	0	3.57	3.75	+0.18	3188	3030	−158	764	772	+ 8
	Significance of differences	none			none			none			none			none		

For legends consult Table I.

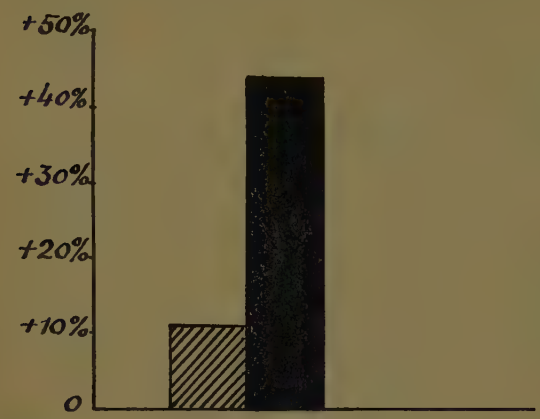

Fig. 1. The increase of systemic and pulmonary arterial systolic resistance in per cent of initial value, following the administration of dihydroergotamine.

altered. The increase in resistance is suggestive of vasocontriction on both sides of the circulation. Though the absolute increase in resistance is equal on both sides, if related to the starting values, a large difference is, however, revealed. While the increase in systemic resistance is 11 per cent only, that in the pulmonary circuit equals 44 per cent (Figure 1). No correlation could be demonstrated between the increase in systemic and pulmonary resistance. (Correlation coefficient = r = + 0.845; t = 1.645; P > 0.10.)

The effect of DHE appeared within 4—5 minutes and could still be demonstrated after an hour. As pointed out in another report Halmágyi et al., (1953), it remains uninfluenced by tetraethylammonium bromide, but drops promptly to normal levels after the administration of sodium nitrite.

No effect was observed following the a ministration of hydergin (Table II). T difference between the effect of DHE a hydergin on pulmonary arterial pressure statistically significant (t = 4.44; P< 0.0

DISCUSSION

DHE-induced pulmonary hypertension caused by a vasoconstriction of the p monary vascular tree. This effect is due a direct chemical effect, similar to that anoxia. It seems that hydergin does n contain this vaso-active principle which m be held responsible for the pulmonary vas constrcition.

It seems obvious that the pulmonary c cuit of normal man is not irresponsive wards pharmacological stimuli. Its respo siveness is, however, qualitatively differe from that of the systemic circulation.

The above effect of DHE seems to ha some practical implications. Its use shou be avoided in impending or manifest rig heart failure.

SUMMARY

The administration of dihydroergotam is followed by a significant increase in p monary arterial pressure and resistance, a consequence of a direct chemical effect the pulmonary vessels, in man. Hyder failed to elevate pulmonary pressure a resistance.

ACKNOWLEDGEMENT

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VARIATIONS IN THE OXYGEN AND CARBON DIOXIDE TENSIONS OF END-TIDAL AIR IN GRADUATED EXCERCIS IN NORMAL PERSONS AND PATIENTS SUFFERING FROM VENTILATORY INSUFFICIENCY

(PRELIMINARY REPORT)

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The composition of the alveolar air during muscular work has been the subject of many studies and discussions (Aitken & Clark-Kennedy, 1928, v. Euler et al., 1939, Asmussen & Consolazio, 1941, Asmussen, Christensen & Nielsen, 1943, Asmussen, Nielsen & Wieth-Pedersen, 1943, Asmussen & Nielsen, 1946, Lilienthal et al., 1946, Galdston & Wollack, 1947, Asmussen & Nielsen, 1950, Nielsen & Smith, 1951). It has been observed that in healthy subjects it is possible, with the automatic alveolar air sampler of Rahn (Rahn & Otis, 1949), to take a sample from the final portion of each expired breath continuously, end tidal air (Rahn), which during moderate exercise approximately follows the changes of the oxygen and carbon dioxide tensions in the arterial blood (Suskind et al., 1950). However, earlier investigations of alveolar air during muscular work have been performed on healthy subjects or on persons not suffering from pulmonary insufficiency Cournand & Richards, 1941). The information obtained from the earlier studies may not be directly applied to patients suffering from pulmonary insufficiency. The division of pulmonary insufficiency into ventilatory, alveolorespiratory, and cardiocirculatory insufficiency (Birath, 1944, Baldwin et al., 1948) contributed much to the physiological unde standing of pulmonary diseases. The obj of this study, the end tidal air in graduat exercise in patients suffering from ventilato insufficiency, was chosen according to t division.

METHODS

Eighteen healthy subjects, three women fifteen men, 19 to 28 years of age, and fift patients, four women and eleven men, 16 to years of age, suffering from some chronic monary disease were tested. The ventilation examined with a modification of the Bened Roth spirometer, to which a ventilograph had b attached. The *Vital Capacity* with its subdivisi and the *Maximal Ventilation* (or Maxim breathing capacity) were determined spirometric and the obtained values corrected to 37° C 760 mm Hg (BTPS). The graduated exer was adjusted with a bicycle ergometer (Dahlstr 1949) using loads of 300 kgm/min., 600 kgm/m 900 kgm/min. and 1200 kgm/min. With diffe loads the exercise was maintained only for minutes, since it proved that particularly th who were seriously affeceed could not carry on longer periods. The exercise was taken at in vals of 5—10 minutes, after the breathing and pulse of the subject had become quite steady. actual ventilation was determined by a gasom during the second minute and the values corre to BTPS. The End Tidal Air samples were ta

h a modification of Rahn's sampler (Rahn & is, 1949) at the end of each exercise and the nples were studied with Scholander's micro-thod (1947) and the values corrected to 0° C l 760 mHg (STPD).

RESULTS

Dyspnoea appeared in the subjects at very fferent phases; in the affected persons, wever, at a lower load than in the healthy bjects. It proved that a division of the aterial according to the value of the *eathing reserve* with different loads was ost appropriate. As it has been observed rlier that dyspnoea will appear if the eathing reserve is smaller than 60—70 per nt of the maximal ventilation (Cournand Richards, 1941) the ratio

$$\frac{\text{Breathing reserve}}{\text{Maximum breathing capacity}} \times 100$$

s used as the basis for the grouping. The subjects in whom this ratio at rest s smaller than 70 (5 patients) belonged the first group. On the second group were aced those in whom this ratio at a load of 300 kgm/min. was smaller than 70 (5 patients); in the third, those in whom the ratio with a load of 700 to 900 kgm/min. was under 70 (5 patients) and in the fourth group those subjects in whom the ratio only at a load of 1200 kgm/min. was equally great, smaller or larger than 70 all the healthy persons. (Table I and Table II.)

The End Tital Air partial pressures (pO_2 and pCO_2) in these different groups seemed to differ from each other, while in the members of each group the oxygen and carbon dioxide pressures went approximately in the same direction, depending on the load. (Table III).

The oxygen pressure (Figure 1) in the healthy subjects seemed to decrease continuously with an increase of the load until the load was 900 kgm/min., when the pressure started to rise. At rest the pO_2 was on an average 110.4 mmHg, after a load of 900 kgm/min. 98.3 mmHg and after a load of 1200 kgm/min. 105.5 mmHg. In the third group there occurred an obvious decrease in the pO_2 up to the load of 300 kgm/min.,

Table I. *The grouping of experimental material according to the ratio* $\frac{\text{Breathing reserve}}{\text{Maximum breathing capacity}} \times 100$ *at different loads. S. D. = standard deviation of ratio.*

Kgm/min.	$\frac{\text{Breathing reserve}}{\text{Maximum breathing capacity}} \times 100$			
	Group I	Group II	Group III	Group IV
Rest	69 S. D. ± 1.7	70 <		
300	65 >	67 S. D. ± 7.1		
500		62 S. D. ± 6.2		
600		60 >	75 <	
700			74 S. D. ± 6.8	
900			69 S. D. ± 3.9	
1200				70 ≦

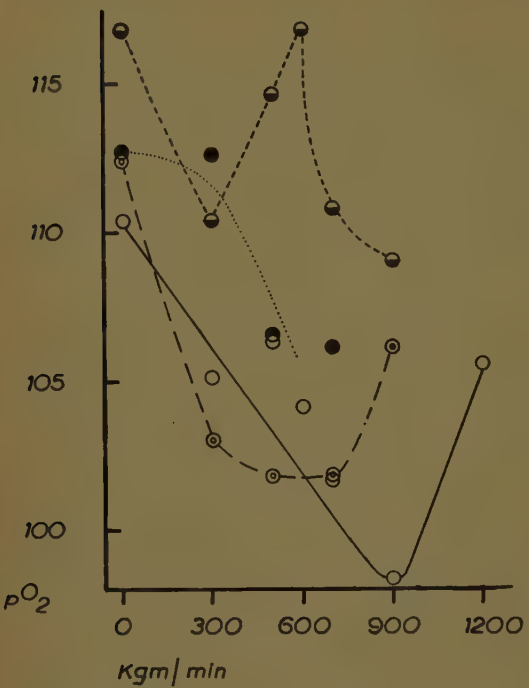

Fig. 1. Relation between mean O_2 tension in end tidal air and the load. ●·····● = group I, in whom the ratio $\frac{\text{Breathing reserve}}{\text{Maximum breathing capacity}} \times 100$ was at rest smaller than 70; ◒-----◒ = group II, in whom this ratio was at a load of 300 kgm/min. smaller than 70; ⊙ ——⊙ = group III, in whom this ratio was at a load of 700—900 kgm/min. smaller than 70; ○———○ = group IV, all normal subjects.

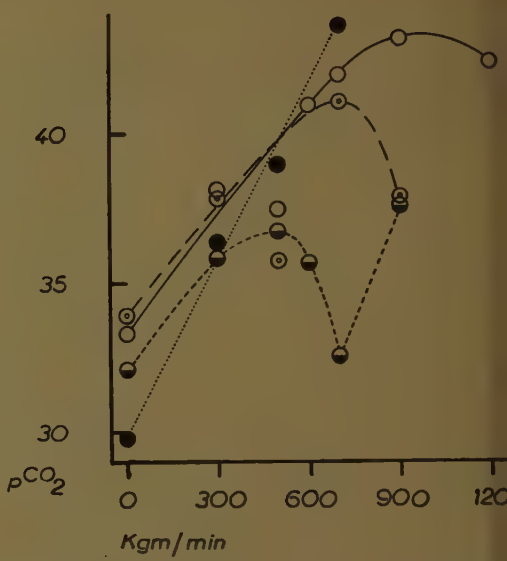

Fig. 2. Relation between mean CO_2 tension in tidal air and the load. For symbols see Fig. 1

after which the decrease became slower, but with a load of 700 kgm/min. there was again an increase in pO_2. In the second group the originally rather high oxygen pressure decreased abruptly already with the load of 500 kgm/min. to decrease as steeply again with the load of 700 kgm/min. In the first group the oxygen pressure still with the load of 300 kgm/min. remained unchanged, but started to decrease sharply after it.

The carbon dioxide partial pressu (Figure 2) were similar, but in reve order. In the healthy subjects at rest pCO_2 was on an average 33.3 mmHg, creasing after that until the load was kgm/min., when it achieved its high value, 43.1 mmHg. With the load of 1 kgm/min. the pCO_2 decreased again some extent. In the third group there an increase in the beginning; the high value (pCO_2 41 mmHg) was achieved w the load of 700 kgm/min., after which th was again a decrease. In the second gr the carbon dioxide partial pressure creased in the beginning, being at highest (pCO_2 36.7 mmHg) at the l of 500 kgm/min., after which it decrea being on an average 32.5 at the load of kgm./min., and after this it increased ag

Table II. *Age, sex and clinical diagnosis of the subjects suffering from pulmonary diseases.*

Group	Subject	Age	Sex	Diagnosis
I	S. T.	33	W	Kyphosis. Asthma bronchiale. Emphysema pulm.
	V. E.	41	M	Emphysema pulmonum
	M. S.	35	M	Tuberculosis pulm. l. a. Pneumothorax l. dx.
	N. A.	53	M	Emphysema pulmonum. Febris rheumatica
	V. P.	20	W	Asthma bronchiale. Bronchitis
II	V. S.	69	M	Bronchiectasiae l. a.
	R. J.	52	M	Bronchiectasiae l. dx.
	E. A.	60	W	Bronchitis asthmatica
	R. H.	42	M	Bronchitis asthmatica
	E. K.	49	M	Asthma bronchiale. Emphysema pulmonum
III	O. L.	60	M	Emphysema pulm. Myodegeneratio cordis
	P. O.	16	M	Bronchitis asthmatica
	O. H.	41	M	Infiltratio pulm. l. dx. Emphysema levis pulm.
	M. J.	51	M	Emphysema pulmonum. Febris rheumatica
	A. P.	44	W	Bronchitis asthmatica

In the first group, however, the originally ther low carbon dioxide partial pressure creased rather sharply all the time with e increase of the load.

DISCUSSION AND CONCLUSIONS

The experimental material of this study small therefore possibly unreliable. In tients suffering from ventilatory insuffici- cy the scattering of the results is large d definite conclusions cannot be drawn. owever, it has been observed earlier that reater differences of the carbon dioxide rtial pressures in the blood during exer- ce are found even in persons who are althy but in a somewhat weak physical ndition than in physically fit subjects Hickam et al., 1951).

The different forms of pulmonary in- fficiency are mostly multiple, so that aterial for pure ventilartory insufficiently is not easily found (Richards, 1950). Also in this study many of the subjects were emphysematic, exhibiting all different forms of pulmonary insufficiency (Baldwin et al., 1949). Particularly the disturbances of the distribution process, which are typical of emphysema (Darling et al., 1944, Fowler & Comroe, 1948, Fowler, 1949) can affect the determination of the alveolar air, especially since it is known that even in normal persons the composition of alveolar air changes depending on the depth of breathing becouse of the unevenness of the distribution process (Nielsen & Sonne, 1932, Grosse-Brockhoff et al., 1936, Roelsen, 1939, Mundt et al., 1940, Sonne, 1934).

Here the interpretation of the analysis of the alveolar air can be disturbed also by several well-known factors such as differences in breathing frequency, possible differences in the dead space of the respiratory passages, the oxygen saturation of the blood and the

Table III.

Group	I			II			III			IV		
End tidal air	pO_2	S. D. ±	pCO_2	pO_2	S. D. ±	pCO_2	pO_2	S. D. ±	pCO_2	pO_2	S. D. ±	pCO_2	S. D. ±
Rest	122.7 (5)	6.2	29.8 (5)	116.8 (5)	5.3	32.1 (5)	112.4 (5)	9.6	33.9 (5)	110.4 (16)	5.2	33.3 (16)	2.8
300 kg/min	112.6 (5)	9.7	36.3 (5)	110.4 (5)	5.3	35.8 (5)	103.0 (5)	6.8	37.8 (5)	105.1 (18)	5.8	38.1 (18)	4.6
500 —»—	106.5 (4)	5.4	38.9 (4)	114.7 (4)	7.7	36.7 (4)	101.8 (4)	9.0	35.7 (5)	106.3 (9)	5.3	37.4 (9)	3.9
600 —»—				116.8 (2)	1.0	35.6 (2)				104.1 (9)	5.8	40.9 (9)	2.6
700 —»—	106.1 (2)	9.2	43.5 (2)	110.8 (2)	4.0	32.5 (2)	101.8 (4)	8.6	41.0 (4)	101.7 (9)	4.52	41.9 (9)	2.2
900 —»—				109.0 (3)	7.8	37.5 (3)	107.1 (5)	11.4	37.8 (5)	98.3 (18)	7.3	43.1 (18)	2.3
1200 —»—										105.5 (11)	9.9	42.3 (11)	5.5

The average pO_2 and pCO_2 in the end tidal air at different loads in each group.
The figures in parentheses show the number of subjets. S. D. = standard deviation.

rate at which the blood loses carbon dioxi
Grosse-Brockhoff & Schoedel, 1936, Scho
del, 1939, Mündt et al., 1940, Batema
1945).

In this study the relatively high values
the oxygen pressures and the relatively lo
partial pressures of carbon dioxide at re
as compared with the values reported
Suskind and his collaborators (1950) mig
in the first place result from hyperventil
tion.

Gray (1950) states that all adjustme
of breathing occurs either in a compensato
or metabolic way; all stimuli of ventilati
are additive according to his multiple fact
theory (Gray, 1945—1946). It appears th
in groups II, III and IV in the present pap
the forms of the curves describe the primar
compensatory multiple adjustment up to
certain load. Still, in different groups
culmination is found in different positio
A similar observation has been made in
healthy subjects in whom, working w
a grou of weaker muscles, the culminati
was achieved earlier (Asmussen & Niels
1946). In this study the culmination w
achieved earlier by those subjects in wh
the ventilatory capacity was lower.

In the same groups, II, III and IV,
increase of pO_2 and the decrease of pC
and the decrease of pCO_2 after the culmi
tion obviously showed the primarily me
bolic multiple part of the adjustment acco
ing to this study.

In group I, however, a continuous decre
was observed in pO_2 and a continuous
crease in pCO_2 as far as it was possible
increase the load. In the second group a n
culmination was observed and after tha
decrease in pO_2 and a increase in pC

hether these are accidental results, or ıether they show a catastrophic inability the ventilatory adjustment, cannot be con-ıded with certainty. Therefore, the inten-n is to continue the investigation, including o other forms of pulmonary insufficiency.

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ELECTROPHORETIC DEMONSTRATION OF TWO TRYPSIN INHIBITORS IN HUMAN BLOOD SERUM

In 1897 Camus & Gley showed that serum hibits trypsin. In 1900 Landsteiner found at the trypsin inhibiting activity of serum as to be found in the albumin fraction. nce then many investigations have been rried out on the nature of the trypsin in- biting substance and on the variation of the ypsin inhibiting activity of normal and thological sera. In 1949 Grob analyzed rum fractions obtained according to Cohn's hanol fractionation method. He found the ypsin inhibiting activity mainly in fractions /: 1 and IV: 4. The sum of the inhibiting tivity of these fractions, however, was only small part of the total inhibiting activity whole serum. On account of the varying ability of the trypsin inhibitor in different uman sera, Todd (1949) suggested that ere might be two different inhibitors.

By electrophoretic fractionation on filter aper I have been able to confirm this hypo- esis and to show that there are two dif- rent trypsin inhibitors in serum, one oving with the α_1 and one with the α_2 obulins.

EXPERIMENTAL

The technique is essentially that of Kunkel Tiselius (1951).

0.1 ml serum is placed on a strip of oistened filter paper (50 × 8 cm) (Munk- ll N: r 20) 10 cm from the end next to e cathode. The paper strip is then inter- osed between two glass plates with its ends pping into the buffer solution (sodium etate 0.029 M; sodium diethyl-barbiturate 032 M. pH adjusted to 8.5 with HCl) of two electrode vessels and 200 volt is applied to the electrodes. The electrophoresis is run for 40 hours. After electrophoresis the paper is cut crosswise into 10 mm broad sections. Each cross section is then eluated 3 times with 0.2 ml acetate-diethyl-barbiturate buffer pH 7.5 and tested for trypsin inhibitor. In order to localize the trypsin inhibiting activity in a common paper electrophoresis diagram another aliquot of the serum is run simultaneously in the same apparatus. This paper strip is colored with bromphenol blue and cut into cross sections in the same way as the paper strip used for the testing of trypsin inhibitor. The color is then eluated and the extinction values are read at 5,750 Å in a Beckman spectrophotometer.

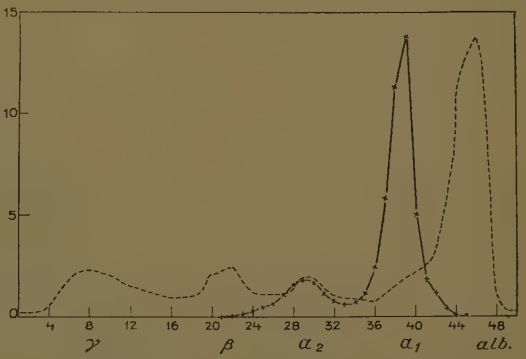

Fig. 1. Localization of trypsin inhibitors in a common paper electrophoresis diagram.
Dotted line: Electrophoretic protein diagram of a normal serum.
Solid line: Electrophoretic trypsin inhibitor diagram of the same serum. Abscissa: Cross section number. Ordinate: γ trypsin inhibited.

Trypsin activities are measured essentially according to Anson (1939).

The diagram of a typical experiment shows the electrophoretic localization of the two inhibitors.

A detailed report will appear later.

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A PHOTOMETRIC SCANNING DEVICE FOR PAPER ELECTROPHORESIS

During the relatively short time which has assed since paper electrophoresis was in-roduced, this method has been used exten-ively in many clinical laboratories as a rou-ne method for examining the protein com-osition of blood plasma etc. After the elec-rophoresis is completed, the strip of paper stained, the quantity of the bound dye eing a measure of the amount of protein in ne various parts of the strip. The quantita-ve analysis may be carried out in two dif-erent ways. The dye in the various cross ections of the paper strip is either de-ermined colorimetrically after elution, or ne optical densities along the strip may be ead directly in a specially constructed photo-neter. The latter method is an essential im-rovement and saves much time, therefore ne need of such a suitable apparatus is bvious.

The photometric scanning device,[1] briefly described as follows, has been constructed to fulfill this requirement. It is based upon the same principles which have been given by Grassman et al. (1951).

The following points, however, are taken into special consideration:

1) It is desirable that the apparatus be simple and practical for routine clinical work.
2) The width of the cross sections which will be photometered should preferably be variable.
3) The light path and shielding must be such as not to permit overlapping or diastasis on the borders of the consecutively photometered areas.

[1] Manufactured by Kelab AB, Stockholm.

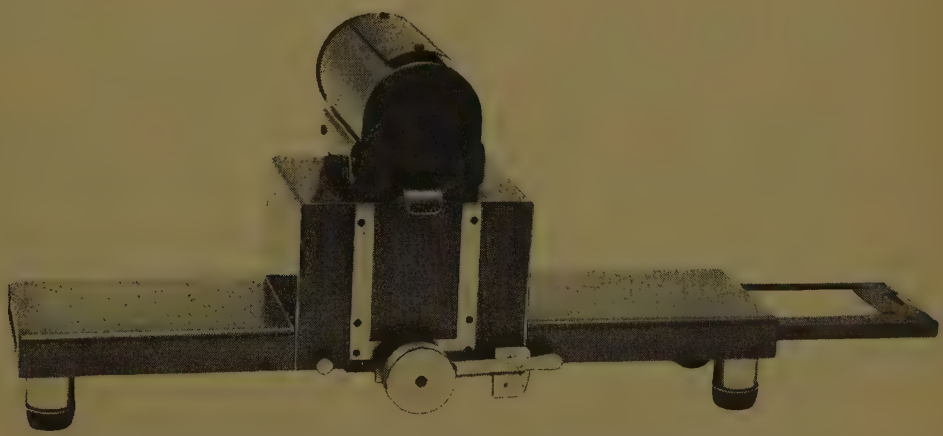

Fig. 1. The Photometric Scanning Device for Paper Electrophoresis.

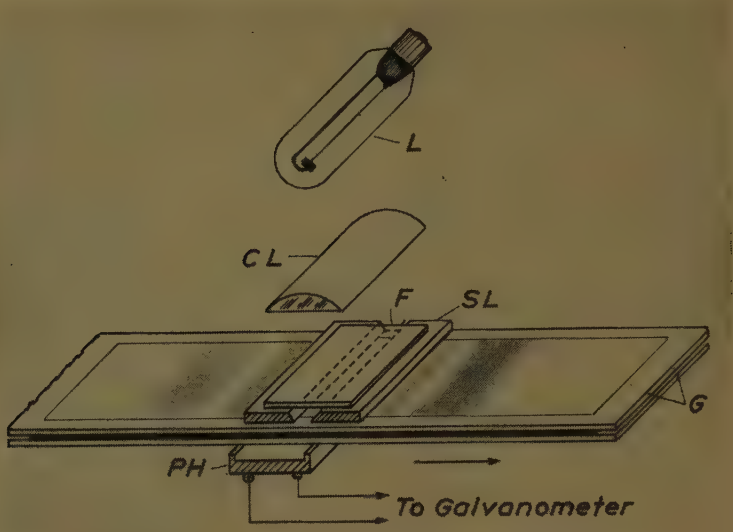

Fig. 2. Schematic drawing of the Photometric Scanning Device.

The appearance of the apparatus is shown in Fig. 1 and its schematic construction in Fig. 2. The paper strip (Whatman 1), which is placed between two glass plates (G) in a metal frame, is moved past a slit (SL) and a filtre (F) in the lightpath between the light source (L) and the photoelectric cell (PH). The movement is carried out step by step with the aid of a special adjustable arrangement with exact spacings of 1, 2, 3 or 5 mm, depending upon the slit used. The light source (L) is a tube shaped lamp, type T3A, with a long and precisely centered filament. The lamp receives the current (1.5 A) from a 6 v storage battery or can be plugged into an ordinary service outlet using a suitable constant voltage transformer to compensate all ordinary fluctuations in line voltage. The light having been refracted by a cylindrical lens (CL) into a parallel beam reaches the slit (SL), which is changeable (1, 2, 3 or 5 mm wide), and thereafter a long selenium photoelectric cell.

The extinction values $\left(E = \log \frac{I_0}{I}\right)$ are read on a suitable galvanometer with có resistance of 20—100 ohms and with sens vity of about 2×10^{-8} A/mm, which is co nected to the photocell. The galvanomet is set by means of the sensitivity knob in ze absorption ($I_0 = I$) over the non-stained, i. non protein-containing, part of the filt paper where the absorption value is lowe The galvanometer readings obtained as t paper is moved along past the slit are plott in a coordinate system, whereby the electr phoresis curve is obtained.

It is to be observed that for a direct phot metric reading the protein solution must placed on the filter paper in the form of even line at right angles to the direction migration, thus the extinction curves o tained approach the ideal Gaussian curv

A good control of the correctness of t optical system can be obtained by exposi a photographic paper which is placed in t frame and fed step by step through the app ratus. Overlapping and diastasis must n occur between the exposed cross sectio and the slit should be uniformly illuminat

Proportionality between light transmission
d the photocell current and respective de-
ction of the galvanometer is controlled
th a linear optical wedge, which is fed
rough the apparatus step by step. This
ntrol proves the linearity of the instru-
ent.

In order that Beer's law would also be
lid for the direct photometry of electro-
oresis strips where the stain intensities are
eatly variable, one must avoid working at
gh extinction values ($E > 0.7$). Conse-
iently a suitable quantity of protein solution
ust be placed on the paper strip to obtain
e extinction values lower than 0.7 for the
otein components.

In instances of direct photometry the
otein should not be stained with an in-
cator dye like bromphenol blue which can
sily change its shade. Instead e.g. "Amido-
hwarz" can be used (Grassman et al.).

Direct photometry is irreplaceable in instances where the dyes used cannot be eluted from the paper strips, as is the case e. g. in the determination of protein-bound carbohydrates (Köiw & Grönwall 1952).

Acknowledgement is made to Dr. G. Wallenius for valuable discussions and suggestions.

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INACTIVATION OF BENZYLPENICILLIN-β-DIETHYLAMINOETHYL ESTER HYDRIODIDE IN THE BLOOD

As shown by K. A. Jensen and associates (1951) the distribution of benzylpenicillin-β-diethylaminoethyl ester hydriodide (PDE-HI) in the animal differs from that of penicillin. Thus, PDE-HI possesses an affinity for the lung tissue for which reason it is used in certain diseases of the respiratory tract. It has been demonstrated also that injection of PDE-HI is followed by a lower and briefer concentration of penicillin in the blood (free penicillin and ester of penicillin) than injection of procaine penicillin in the same dose. This phenomenon, which cannot be due to the affinity of the drug for certain organs, has also been observed in our laboratory. Quantitatively it appears to vary from species to species. Experiments including determination of the drug in body fluids have shown, however, that there is a marked difference from one species to another in the capacity of the blood to inactivate PDE-HI. Since data of this kind are of considerable significance in evaluating the results of animal experiments, primarily with regard to the possibility of drawing analogous conclusions from one species to another, a few preliminary results will be reported.

Dissolved in water, PDE-HI is hydrolyzed to penicillin and diethylaminoethyl alcohol. The process depends on the hydrogen ion concentration and the temperature. In a 0.9 per cent sodium chloride solution, at pH 7.3 and 37° C it undergoes 50 per cent hydrolysis within 23 minutes (Jensen et al., 1951). Following hydrolysis, the liberated penicillin may be assayed biologically using sodium penicillin as a standard by the usual plate method. When assayed thus, 1 mg PDE-HI corresponds to about 1000 I.U. sodium penicillin. This is in accord with t stoechiometric relations.

The following experiments were conduct to study the possibility of recovering pe cillin and PDE-HI in the blood:

PDE-HI was added to citrated blo samples in quantities to make 50 μg per m The samples were allowed to stand at 37° for one hour. Then the penicillin conte was determined after suitable dilution of t sample with a phosphate buffer pH 7.2. this procedure, the amount of PDE-H added should give a penicillin content cor sponding to 50 I. U. per ml.

The same experiments were made wi sodium penicillin in concentrations of 50 I per ml. Moreover, control experiments we

Table I. *Recovery of PDE-HI adde to blood.*

Dissolved in blood from	I. U. of penicillin recover after addition of			
	PDE-HI, 50 μg per ml		Penicillin 50 I. U. per	
	i. u.	per cent	i. u.	pe ce
Humans	31	62	54	10
Monkeys	25	50	46	9
Rabbits	13	26	49	9
Guinea-pigs	4.7	9	49	9
Rats	13	26	42	8
Hamsters	15	30	52	10
Mice	41	82	47	9
Dissolved in buffer	49	98	52	10

arried out with PDE-HI and sodium peni-
llin dissolved in a phosphate buffer pH 7.2
nstead of blood.

Each of the data shown in the table is the
verage values of 10 experiments with 10
ifferent blood samples. The values for
nonkey blood, however, are derived from
nly two different blood samples.

In the samples with sodium penicillin,
enicillin was recovered almost quantita-
vely. On the other hand, only part of the
mount of PDE-HI added could be re-
overed. An exception was mouse blood in
hich 82 per cent was recovered as peni-
llin. As far as the other blood samples are
oncerned, the recovery percentage varied
ithin wide limits from species to species,
ecreasing in the following order: man, mon-
ey, hamster, rabbit, rat, guinea-pig.

This inactivation, which is most marked in guinea-pig blood in which less than 10 per cent of the amount was recovered, must be taken into consideration in all studies on the pharmacology of PDE-HI.

Studies currently in progress (which will be reported later) indicate that this inactivation is due in part to plasma proteins and in part to the blood cells.

The reason why mouse blood does not inactivate PDE-HI may be that in the blood the ester undergoes enzymatic hydrolysis with liberation of penicillin (Milthers & Roholt). In mouse blood, therefore, PDE-HI behaves like penicillin.

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Milthers, K. & Roholt, K.: To be published.

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AN IMPROVED REAGENT FOR OXYGEN ABSORPTION IN GAS ANALYSIS

With the Haldane apparatus for gas analysis many reagents for oxygen absorption have been proposed for use but most of them have disadvantages with regard to rate of absorption, stability of solution, absorption capacity, or sensitivity to other gases. The substance described in this note is considered to be the most versatile yet used for oxygen absorption, its only disadvantages being the necessity for removal of carbon dioxide from gases before oxygen analysis since the absorbent is used in an alkaline medium.

The substance is 1:2:4-triacetylhydroxyhydroquinone (1:2:4-triacetoxybenzene) and it is quite stable as a pure solid. For use as an oxygen absorbent 10 g of the compound, 15 g of potassium hydroxide and 100 ml of distilled water are shaken up in a stoppered flask until a clear deep red solution is obtained. After cooling, the solution is ready for use in the usual way.

Like pyrogallol, 1 g-mol. absorbs 1.5 g-mol. of oxygen but unlike the former the new absorbent does not tend to evolve carbon monoxide during oxygen absorption. It has been found that usually only four or five washings are required for complete absorption instead of the eight washings required for the chromous chloride reagent (Dahlström & Wahlund 1949). As the reagent becomes inactive the colour gradually changes to a deep chocolate brown but the capacity and rate of absorption remain very high until the reagent is almost inactive.

The compound can be purchased inexpensively as a dark brown amorphous powder from certain drug houses or it can be prepared in the laboratory by means of the Thiele reaction. In this preparation quinone reacts with acetic anhydride under catalytic influence of sulphuric acid to give an additive product which isomerizes, with further acetylation, to give 1:2:4-triacetoxybenzene.

Gas analysis involving hydrogen can be made in apparatus containing the improved reagent and in practice it has been found to be far superior to other absorbents. It is of interest to note that in a recent mention of the compound it is designated as Pyrogallol A. (Anon 1952.).

Bedford, England.
Jan. 30, 1953.

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COMMENTS REGARDING THE EVALUATION OF PAPER ELECTROPHORESIS OF PROTEINS

The results obtained with paper electrophoresis of serum proteins are often compared with those obtained with free electrophoresis, but in order to get reasonable accordance it may be necessary to multiply the globulin values with a factor. Different values have been reported for this factor, and this seems to depend upon differences in the methods used. Some of the conditions significant in this connection are mentioned below. In pathological conditions the value of the factor may probably vary although the same technique is employed (Köiw, Walenius & Grönwall 1952), but this will not be discussed here.

1. It is obvious that a shift in the level of the base line will alter the areas corresponding to the different protein fractions and also their relative values (Sommerfelt 1952). Different criteria for the location of the base line may therefore account for some of the discrepancies in the value of the factor. It is recommended to register many blank values on both sides of the protein and use the mean of these blanks as the base line.

2. At present bromphenol blue (Durrum 1950, Cremer & Tiselius 1950) and amidoschwarz 10 B (Grassmann & Hannig 1950) seem to be the dyes most widely used. In the literature it is often stated that bromphenol blue is to be preferred in the elution method, because it is easily extracted from the paper strips. With this procedure, however, it is necessary to use a factor (most investigators state 1.5 or 1.6) in order to get the globulin values in reasonable accordance with those obtained by free electrophoresis. Staining in exactly the same manner, but with evaluation of the strips by direct photometry, gives results very similar to those obtained with free electrophoresis, without the application of any factor. (This was suggested by Köiw and Grönwall.) — Further, if one submits pure protein fractions to an ordinary paper electrophoresis run, followed by bromphenol blue staining and elution of the dye, the curve for gamma globulin will run lower than that for albumin. On the other hand, on direct photometry of pure protein fractions, the curves for gamma globulin and for albumin are very close (Fig. 1). — These observations indicate that the bromphenol blue dye is not easily and uniformly extracted from the strip.

3. With amidoschwarz 10 B followed by elution, it is also necessary to use a factor for the globulins (approximately 1.3). With

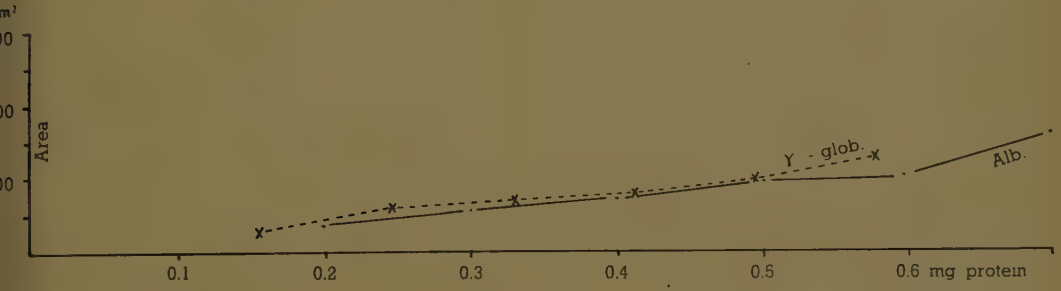

Fig. 1. Bovine plasma albumin and gamma globulin (Armour) run with Grassmann & Hannig's technique, but stained with bromphenol blue.

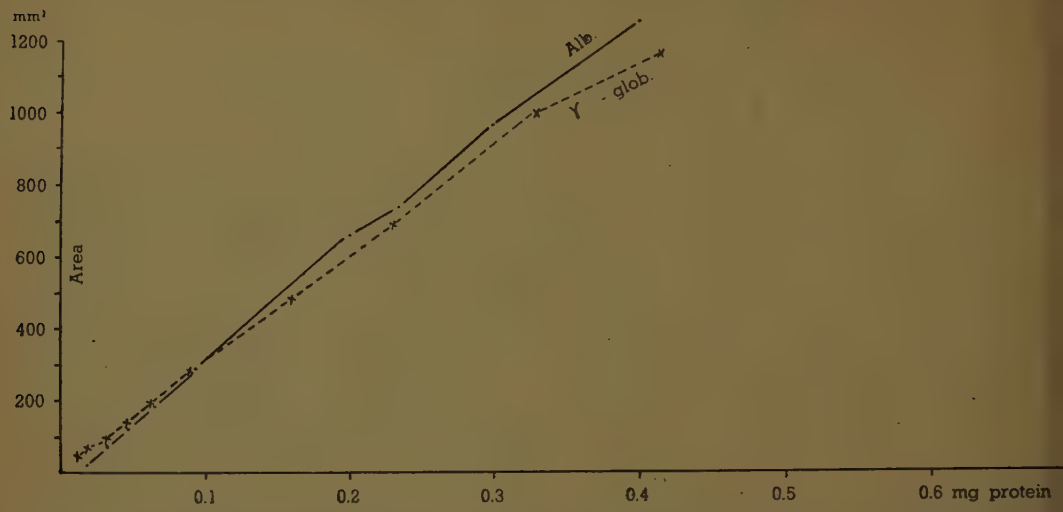

Fig. 2. Bovine plasma albumin and gamma globulin (Armour) run with Grassmann & Hannig's technique, stained with amidoschwarz 10 B. Each point indicates the mean of 2—4 analyses.

the same dye and direct photometry (Fig. 2), no factor is used.

4. If one compares Fig. 1 and Fig. 2 it is impressive that the curves for bromphenol blue staining run very flat. They may of course be made steeper by altering the scale, but in any case they indicate that bromphenol blue is not a satisfactory dye in paper electrophoresis when very small amounts of protein are applied (0.05 ml of serum or less).

5. Although in Fig. 2 the curve for albumin is a little steeper than that for gamma globulin, the difference between them is small. This is partly a result of a shift to the right of the albumin curve, probably caused by the adsorption of albumin to the filter paper during the electrophoretic run (Sommerfelt, to be published). The slight bending of the curves in the upper parts is perhaps caused by technical insufficiency of the method for direct photometry (Grassmann & Hannig 1952). — Human serum protei fractions seem to behave in a similar manne (Sommerfelt, to be published).

Central Laboratory,
University Hospital,
Uppsala, Sweden, and
Central Laboratory,
Drammen Hospital,
Drammen, Norway.
Jan. 15. 1953.

S. Chr. Sommerfe

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Book Review

ANS SELYE: *Annual Report on STRESS (1950—51).* Acta Inc. Montreal. 511 p. 1951.

The tremendous working capacity of the cham-
on of the stress concept, Hans Selye, has allowed
n to publish shortly ofter the edition of his well-
own monography on Stress, another book which
unique in several respects. First of all, it is
treatise and not only an abstract based on some
00 references from the litterature 1948—51.
condly, it contains in concentrated writing, the
adlights of the general physiology and pathology
stress which enables every doctor to grasp the
ain ideas and the philosophy of the stress con-
ot as it is formulated anno 1951.

The second part of the book contains experi-
ental results and clinical observations, as inter-
eted by Selye, from the colossal body of medical
erature from the last 4 years on the special physio-
gy and pathology of stress. What makes this sec-
n, and the book itself, so valuable to all workers
this vast field, is the index system used which
gether with the immense list of references enables
eryone interested in a particular field, however
rrow, to find without loss of time what he is
oking for, in the text as well as in the refer-
ce list.

Selye possesses an abundance of practical sense
d is a brilliant salesman. He presents stimulating reading and is fully aware of the impossibility of correlating critically all pertinent facts in an impersonal way when the author himself is so involved in the subject. Some will probably disagree with several of his conclusions on special topics. One should, however, remember that the whole concept of stress is under rapid development and that much which to-day is medical philosophy, may tomorrow be generally accepted knowledge, based on irrefutable evidence.

The third part of the book contains Selye's attempt to sketch a unified theory of medicine. Premature as it may seem, nothing is lost by trying an unification at the present time where medicine in the broadest sense is a mosaique of specialities. The mass of detailed knowledge accumulated in each field is so overwhelming that no single person can extricate himself from the chains of his own specialty and have surplus energy to absorb current ideas in other fields in order to arrive at a unified concept of disease.

Selye has made a bold attempt at this, and he will undoubtedly in the years to come continue to influence medical thought throughout the world.

One will await the forthcoming Annual Report 1952 with impatience.

The printing is excellent, so are the illustrations. Every clinic and laboratory should have these reports on their shelf.

O. J. M.

FIRST WORLD CONGRESS ON FERTILITY AND STERILITY

The First World Congress on Fertility and Sterility will be held on May 25—31, 1953, at the Henry Hudson Hotel in New York City. This Congress is sponsored by the International Fertility Association with the cooperation of the American Society for the Study of Sterility.

Twenty-three scientific sessions are to be held, which will embrace the entire field of fertility and sterility, including sessions dealing with socio-economic factors, psychosomatic aspects, and artificial insemination. The sessions will be conducted in English, French and Spanish, with the use of earphones and simultaneous translations, as in the United Nations meetings.

In addition to the scientific sessions there will be medical round table discussions, question and answer periods, scientific exhibits and motion pictures.

It is anticipated that 1800 scientists from 51 countries will attend the Congress, making it the world's largest medical meeting devoted to problems of reproduction. This Congress will facilitate the exchange of ideas and information among doctors from the various countries, dealing with the very latest findings in fertility studies.

Since seats at the Congress will be at a premium, it is suggested that those who plan to attend write as soon as possible to the Chairman of the Local Arrangements Committee, 1160 Fifth Avenue, New York 29, New York, for advance registration.

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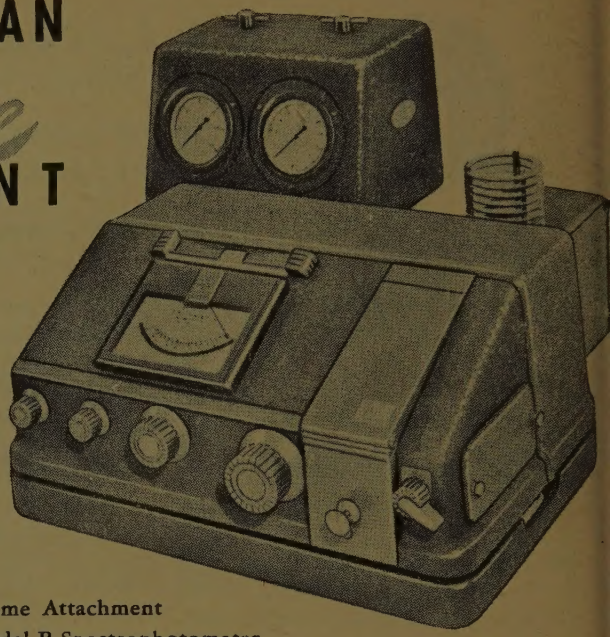

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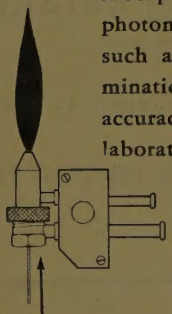

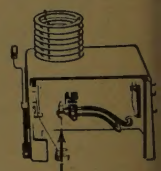

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Manuscripts should be typewritten in English, double spaced throughout.

Tables, diagrams and other inserts should be sent on separate sheets, bearing the author's name. e legends for the figures should be typed separately, and the figures should be comprehensible without erence to the text.

All figures and illustrations are to be submitted in such form as to permit photographic reproduction hout retouching or redrawing. Line drawings should be carefully drafted with black India ink on ite drawing paper or faintly bluelined paper.

The introduction should contain a clear statement of the purpose of the investigation and its ation to other work in the same field, but extensive reviews must be avoided.

The experimental procedure must be clearly described. However, methods should be given in enso only if they represent a new approach.

Every paper should end with a brief summary. References to the literature should be listed habetically at the end of the paper, and the names of the authors should be mentioned in the text with year of publication in brackets. The bibliography should conform to the style used in this journal: e author's name, initials, title of paper, abbreviation of journal, volume, page and year; in the case books, the title in full, with the place and year of publication, and page.

No paper should exceed 12 printed pages. — Short reports of important findings, occupying not re than 2 printed pages, will be accepted for prompt publication in the form of *Letters to the Editors.*

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Jean Mette a/s

Oslo, Tordenskjoldsgt. 3

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